PREPARATION OF SERICIN BASED WOUND DRESSING AND INVESTIGATION OF ITS BIOMATERIAL PROPERTIES

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ABSTRACT

PREPARATION OF SERICIN BASED WOUND DRESSING AND INVESTIGATION OF ITS BIOMATERIAL PROPERTIES

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In this study, it was aimed to produce sericine/collagen composite membranes and to investigate their properties as a wound dressing. Different membrane compositions were prepared by casting and solvent evaporation method. After initial studies for optimization of ratios, membrane groups at two different thicknesses were prepared for each selected ratio and cross-linked with 3 % (w/v) glutaraldehyde (GTA). Considering the wound dressing requirements, equilibrium degree of swelling (EDS), water vapor transmission rate (WVTR), oxygen permeability, mechanical properties, in situ degradation, microbial penetration and cytotoxicity of membranes were examined. The EDS of membranes had a range of 14.91 to 4.37 (g/g) and increased significantly with the presence of sericin. There was no obvious relationship between the sericin ratio of membranes and WVTR, but the increase in membrane thickness

decreased WVTR significantly. Thin and sericin containing membranes had statistically better oxygen permeabilities. Sericin deteriorated the tensile strength and elongation of membranes statistically. Cross-linked groups were resistant to hydrolytic degradation through 4 weeks of incubations. None of the membranes were penetrable to bacteria owing to their dense structure. For cytotoxicity studies, 3T3 fibroblasts and keratinocytes were seeded on membranes separately, and analyzed with MTT assays, and light microscopy and scanning electron microscopy (SEM). As regards to MTT assay, keratinocytes proliferated significantly on membranes and reached to high confluence within 7 days. Similarly, fibroblasts also showed high proliferation on membranes. Light microscopy and SEM analysis showed that both cells could attach, grow and spread on membranes. Also, cells gained their characteristic morphology after 1 day and formed flattened structure within 7 days.

Keywords: Sericin; Collagen; Wound dressing; Membrane

ÖZ

SERİSİN BAZLI POLİMERİK YARA ÖRTÜSÜ HAZIRLANMASI VE BİYOMALZEME ÖZELLİKLERİNİN İNCELENMESİ

Aktürk, Ömer

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Şubat 2009, 112 sayfa

Bu çalışmada, serisin ve kolajen karışımından oluşan kompozit membranlar geliştirmek ve bu membranların yara örtüsü özelliklerinin araştırılması amaçlanmıştır. Farklı membran kompozisyonları, döküm yapılarak ve çözücüleri uçurularak hazırlanmıştır. Membran kompozisyonlarının ilk optimizasyon çalışmalarından sonra, iki farklı kalınlıktaki gruplar her bir kompozisyon için hazırlanmış ve % 3 (w/v) glutaraldehit ile (GTA) çapraz bağlanmıştır. Bir yara örtüsü için gerekli özellikler düşünülerek membranların denge su tutma derecesi (EDS), su buharı geçirme hızı (WVTR), oksijen geçirgenliği, mekanik özellikleri, in situ bozunurluğu, mikrobiyal geçirgenlik ve sitotoksisite özellikleri incelenmiştir. Membranların EDS değerleri 14,91 ve 4,37 (gr/gr) aralığında bulunmuş ve serisin varlığıyla anlamlı arttığı görülmüştür. WVTR ile membranlardaki serisin oranı arasında açık bir ilişki bulunamamıştır; ama membranların kalınlık artışı WVTR değerlerini istatistiksel açıdan düşürmüştür. Serisin içeren ince membranlar daha iyi oksijen geçirgenliğine sahiptir. Serisin, membranların gerilim kuvveti ve uzama gibi

özelliklerini istatistiksel olarak kötüleştirmiştir. Çapraz bağlanan membran gruplarının hidrolitik bozunmaya karşı 4 haftalık inkübasyon süresi boyunca dayandığı görülmüştür. Membranların sıkı yapıda olmasından dolayı grupların hiçbirisinin bakterilere karşı geçirgen olmadığı görülmüştür. Sitotoksisite çalışmaları için 3T3 fibroblastlar ve keratinositler membranlar üzerine ayrı ayrı ekilerek MTT hücre canlılığı tahlili yapılmış, ışık mikroskobu ve taramalı elektron mikroskobu (SEM) ile analiz edilmişlerdir. MTT analizine göre, keratinositler 7 gün içinde istatistiksel olarak önemli ölçüde artmış ve yüksek konfluansa ulaşmıştır. Benzer şekilde fibroblastlar da membranlar üzerinde iyi bir şekilde artmıştır. Işık mikroskobu ve SEM analizleri her iki hücrenin de membranlara yapışabildiğini, membranlar üzerinde büyüyebildiğini ve yayılabildiğini göstermiştir. Ayrıca her iki hücre de karakteristik hücre morfolojilerini bir gün sonunda kazanmış ve 7 gün boyunca yassılaşmışlardır.

Anahtar Kelimeler: Serisin; Kollajen; Yara örtüsü; Membran

To my beloved grandmother who has always seen me as a benevolent person and appreciated me for my virtues

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I need to thank all the lab fellows who helped me so much during my fleeting studies in the Membrane Production Laboratory of London Imperial College, Chemical Engineering and Chemical Technologies Department. My study in England was financially supported by British Council. I would like to thank TÜBİTAK for project support via project number of 106M062.

The Last but not the least, I would like to give my special thanks to my parents whom have contributed to the success of this thesis study, maybe even more than I have. I have always them besides me giving their emotional foster and encouraging me whenever I live harsh moments and fall into disappointment in my studies.

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LIST OF ABBREVIATIONS

ANOVA	: Analysis of Variance
ASTM	: American Society for Testing and Materials
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl Sulfoxide
DO	: Dissolved Oxygen
EB	: Elongation at Break
EDS	: Equilibrium Degree of Swelling
EDTA	: Ethylene Diamine Tetra Acetic acid
E	: Modulus of Elasticity
FBS	: Fetal Bovine Serum
GPa	: Giga Pascal
GTA	: Glutaraldehyde
kDa	: Kilo Dalton
MPa	: Mega Pascal
MTT	: Thiazolyl Blue Tetrazolium Bromide
MW	: Molecular Weight
NMF	: Natural Moisture Factor
OD	: Optical Density
PBS	: Phosphate Buffered Saline
RGD	: Arginine-Glycine-Aspartic Acid
RH	: Relative Humidity
SD	: Standard Deviation
SEM	: Scanning Electron Microscope
TCPS	: Tissue Culture Polystyrene Dishes
UTS	: Ultimate Tensile Stress
UV	: Ultraviolet
WVTR	: Water Vapor Transmission Rate

CHAPTER 1

INTRODUCTION

Introduction part of this thesis primarily aims to present the concept of wound dressings as a biomaterial by describing the general skin properties, skin wound healing processes and "properties of wound dressing biomaterials" in detail. Then, elaborate information on two proteins, collagen and sericin is given to fully understand the reasons for why they have been used to produce a composite wound dressing material in the present study. In the last part of the introduction the objectives of the thesis study and the test methods performed to evaluate the required properties were summarized.

1.1. Skin

1.1.1. Anatomy of Healthy Skin

Skin is considered as the largest organ of the body. Anatomically, skin is divided into three main regions, dermis, epidermis and subcutaneous fat layer (http://www.umm.edu/dermatology-info/anatomy.htm).

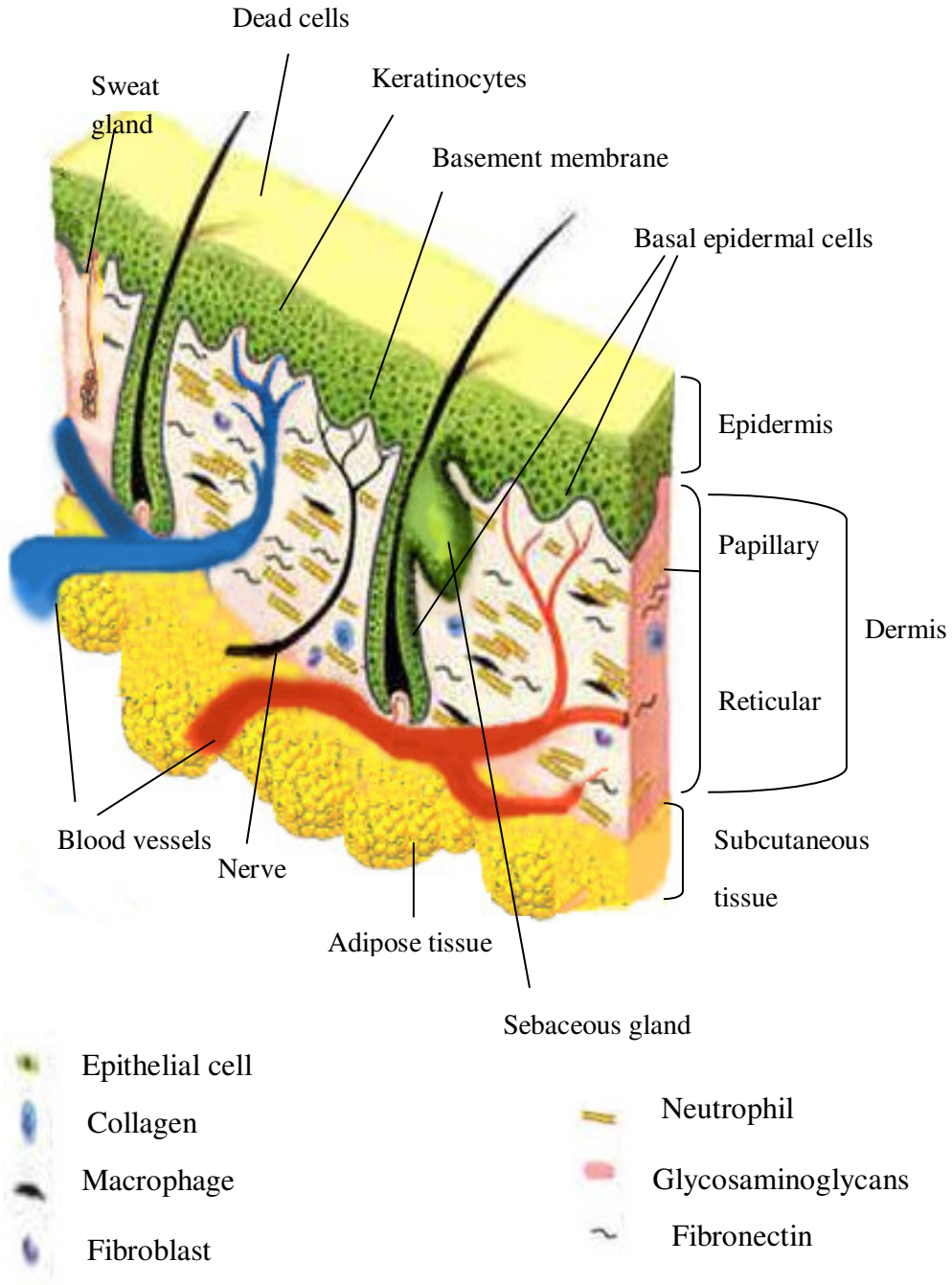


Figure 1.1. Anatomy of skin (adapted from http://www.burnsurgery.org /Betaweb /Modules/skinsubstitutes/sec1.htm)

The outer layer of skin, epidermis, varies in thickness throughout the body such that it is the thinnest (0.05 mm) on eyelids and thickest (1.5 mm) on the palms and soles. The single layer basal epidermal cells which are present in the innermost layer of epidermis continually proliferate and differentiate into keratinocytes and push already formed cells into upper levels. They finally formes flattened, death cells composed of skin proteins called keratins. It does not contain any blood vessels but the nutrients vitamin and oxygen comes from the deeper layers of epidermis with diffusion. At the bottom of epidermis exists a very thin membrane called basement membrane. The boundary between dermis and epidermis contains undulations called rete pegs, which increase the contact area between the layers of skin and aid in preventing the epidermis from being torn. The deeper portion of skin, dermis, also varies in thickness; it is 0.3 mm on eyelids and 3 mm on the back. It contains blood vessels, nerves, and skin appendages such as hair roots, sweat and sebacious glands. Subcutaneous fat made-up of fat-filled cells (adipose cells) underlies this layer. It contains larger blood vessels and nerves and is loosely attached by connective tissues to the muscles and bones. (http://dermatology.about.com/cs/skinanatomy/a/anatomy.htm; http://www.pg.com/science/skincare/Skin_tws_10.htm).

1.1.2. Functions of Skin

Briefly, the functions of skin involve thermoregulation, protection from heat, light and injury, sensation, prevention the entry of bacteria and water loss and storage of water, fat and vitamin D. The overall functions of skin with regards its different layers are described in Table 1.1.

Table 1.1. Functions of skin layers (http://www.pgbeautyscience.com/functions-of-the-dermis.html; http://www.burnsurgery.org/Betaweb/Modules/skinsubstitutes/sec1.htm; Falanga and Faria, 2007)

Epidermis	Dermis
• Prevention of excess water vapor loss • Protection from toxins, heat, UV light and microbial entry • Protection from desiccation • Social-interactive	• Mechanical protection (strength, flexibility and elasticity of skin) • Factory for all the components required for repair and remodeling of epidermis and dermis • Scaffolding for cell migration • Conduit for nutrient and oxygen delivery • Thermoregulation through control of skin blood flow and sweating • Sensation (touch, pain, heat and cold)

1.1.3. Functional Components of Skin

Skin is a multi-component composite of cells and macromolecules (Geesin and Berg, 1991). The major cellular component of epidermis, keratinocytes, forms overlapping structures held together by desmosomes which provide cell to cell adhesion. Dermis is composed largely of extracellular matrix components including collagen, elastin, fibrillin, hyaluronic acid and proteoglycans. Collagen fibers give shape to the skin and prevent premature mechanical failure. Elastin fibers composed of fibrillin and elastin, are responsible from elastic recovery of skin after removal of a mechanical load.

Fibroblast is the most prevalent cell type in skin and is responsible for synthesizing and depositing collagen fibers. This cell type is also responsible for recognition, removal and turnover of proteins that are damaged or are being recycled (Silver, 1994).

Cellular and matrix components of skin with their functions are summarized in Tables 1.2-1.3. (http://www.burnsurgery.org/Betaweb/Modules/skinsubstitut/sec1. .htm).

Table 1.2. Epidermal components of skin

Epidermis
• Outer cells: Keratinocytes
• Keratin: A tough protein on surface, preventing bacteria or toxin entry
• Inner layer: Epidermal cells which are proliferating and migratory to surface and will become keratinocytes.
• Innermost layer: Basal epidermal cells anchored to basement membrane by adhesion molecules.
• Skin appendages anchored in dermis are also lined by epidermal cells.
• Langerhans' cells: The frontline defense of the immune system in the skin.
• Melanocyte: Pigment production.

Table 1.3. Dermal components of skin

Dermis

- Papillary dermis: Upper dermis containing rete pegs, the most biologically active part of the dermis.
- Reticular dermis: The thicker deeper portion responsible for durability and anchoring of skin appendages.
- Matrix proteins:
  - ✓ Collagen is the predominant protein; mainly collagen type I.
  - ✓ Fibronectin is the primary adhesive protein playing a major role in healing.
  - ✓ Other adhesive proteins.
- Ground substances (glycosaminoglycans):
  - ✓ Carbohydrate protein complexes.
  - ✓ Hyaluronic acid.
- Cells:
  - ✓ Fibroblasts.
  - ✓ Macrophages.
  - ✓ Platelets.

1.1.4. Mechanical Properties of Skin

Mechanical properties of skin are largely a result of collagen and elastin fiber networks that form a scaffold within which cells sit. Collagen is the main source of mechanical strength of skin, but elastin has an outstanding elastic property. The surface layer of skin looks creased under visual observation and there exists an internal tension which makes the skin taut. In areas of the body where skin covers the joints, the skin is exposed to high stresses where it must be able to resist.

The mechanical properties of skin are also related with proteoglycans that are found between neighboring collagen fibrils. The epidermis contributes very little to these properties except in areas of the body where it is thick, such as the palms of the hands and soles of the feet.

Collagens (type I and III) form mixed fibers in skin as cross-linked continuous networks that prevent premature mechanical failure.

A typical stress-strain curve for skin obtained from a tensile test illustrates an increasing slope with increasing strain (Figure 1.2). The initial low modulus region involves the removal of undulations and initial alignment of the three dimensionally organized collagen fibers along the tensile load directions. When the collagen fibers bear the full load, a linear stress-strain relationship occurs. At the final stage of test, the skin fails, but skin is believed to operate in the first and second regions physiologically (Silver, 1994; Meyers et al., 2008).

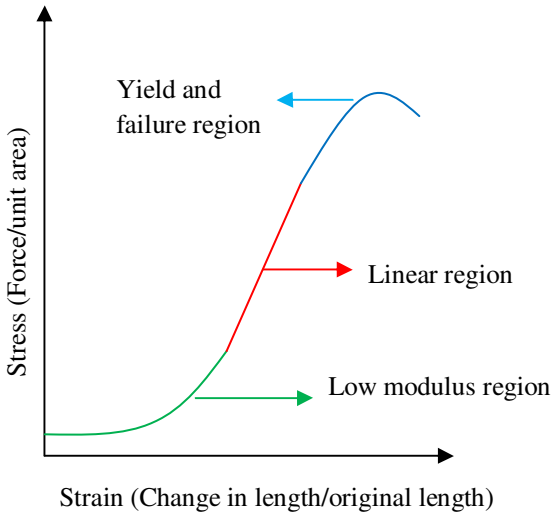


Figure 1.2. A typical stress-strain curve for wet skin (adapted from Silver, 1994).

1.1.5. Repair of Skin

Repair of skin is initiated by mechanical, chemical, bacteriological, viral and other traumatic stimuli (Table 1.2).

Table 1.4. Events that trigger skin repair (Adapted from Silver, 1994).

Event	**Effect**
Bacteriological or viral infection	Inflammatory response
Chemical burns	Degradation of extracellular matrix
Electrical burns	Tissue necrosis
Mechanical trauma	Vascular leakage and tissue swelling

Before the epidermal repair dermal repair involving inflammation, immunity, blood clotting, platelet aggregation, fibrinolysis and activation of complement and kinin systems (Table 1.5) takes place.

Table 1.5. Biological systems-processes involved in skin repair (Adapted from Silver, 1994).

System	Function
Blood clotting and Platelet aggregation	Prevents excess bleeding, Plugs leaks in vessel walls
Complement	Involved in lysis of foreign cells and vasodilatation of vessels
Fibrinolysis	Removes blood clots
Immunity	Destroys foreign bacteria
Inflammation	Cleans up dead tissue
Kinin	Involved in vasodilation

Briefly, tissue repair consists of inflammation, proliferation, granulation and remodeling phases (Figure 1.3). When the blood vessels are disrupted, blood clotting and platelet aggregation systems are activated forming an insoluble fibrin network. A complex of hyaluronic acid bounded to fibrin is synthesized first. This complex forms a matrix which is thought to play a role in the granulation and remodeling phases of wound healing. Then fibronectin is cross-linked to fibrin in the wound. This complex plays an important role in the attraction, migration and adhesion of inflammatory cells such as polymorphonuclear leukocytes. Factors influencing the migration and proliferation of cells that synthesize extracellular matrix are secreted by inflammatory cells. Also, the flow of additional inflammatory cells to the wound is caused by inflammatory cells. Therefore, the first phase of wound healing, inflammation, aids to recruit the inflammatory cells into the wound area and dead tissue is removed by these cells.

The inflammatory phase of wound healing is pursued by a proliferation period in which the cells forming new blood vessels and the components of extracellular matrix proliferate. Fibroblasts attracted by fragments of collagen as well as fibronectin in the blood clot synthesize types I, III and V collagens and

proteoglycans. Eventually, a granulation tissue that is composed of thin randomly organized collagen fibrils is formed immediately after wounding.
Following the granulating phase of dermal wound healing, remodeling of granulation tissue as a result of replacement of random collagen fibrils with oriented large diameter collagen fibrils occurs. The removal of collagen fibrils may follow two possible mechanisms; one by phagocytosis of collagen fibrils, another one is through the removal of collagen fibrils with collagenase synthesized by fibroblasts.

As a subsequence of dermal repair, epidermal migration believed to be facilitated by factors such as fibronectin, epidermal growth factor, interleukin-1 as well as smooth muscle cell factor begins (Silver, 1994; Li et al., 2007; Kondo, 2007).

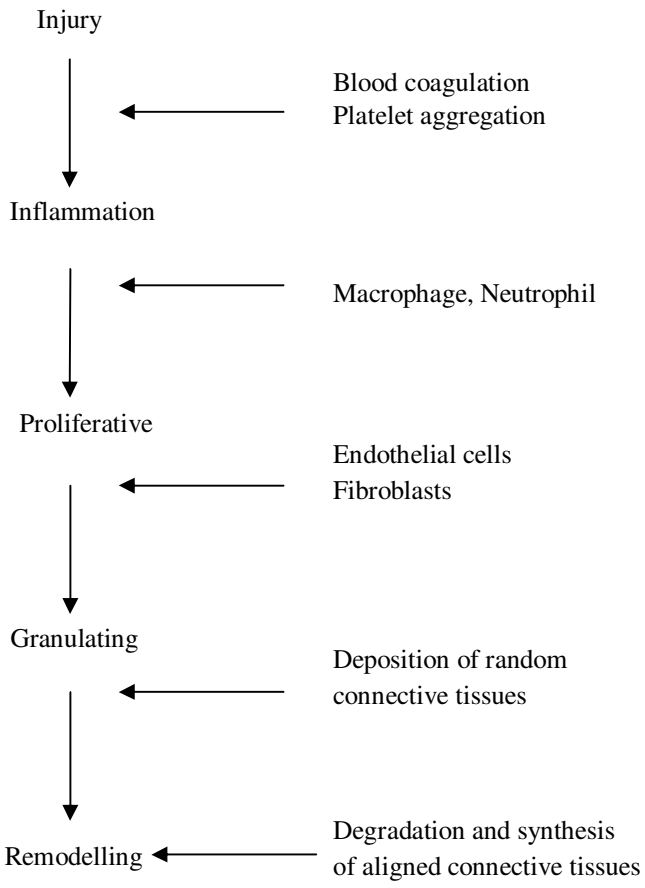


Figure 1.3. Schematic diagram illustrating events that precede healing. Adapted from Silver (1994).

1.2. Wound Dressings

Minor wounds to the skin are encountered every day and these wounds heal themselves in time only causing some pain. Yet, major injuries resulting in extensive damage to the skin requires immediate coverage to aid repair and regeneration to restore normal skin function. Severe acute and chronic wounds such as, burns, mechanical trauma, pressure and leg ulcers, congenital skin diseases and cancer excision pose a great challenge to the surgeons, plastic surgeons and all others associated in this mission. Especially burns and skin ulcers (pressure ulcers and leg ulcers) is a problematic issue. For instance, in the United States, where greater than 1 million burn injuries occur each year, fires and burns results in 45,000 hospitalization and 4500 deaths annually. As regards the chronic wounds, in the United States alone, these wounds are estimated to affect more than 2 million people with total treatment costs as high as $ 1 billion annually. If it is viable the ultimate treatment method performed to get the most aesthetic and functional result for these wounds is autografting. However, between the time of hospitalization and the subsequent preparation of grafting, the wound is covered with an ideal wound dressing to primarily prevent the bacterial invasion and to provide adequate gaseous exchange. Research and development in the field of initial wound coverage before the final autografting has resulted in the fabrication and the production of a wide variety of synthetic and biological dressings. In case of extensive skin loss injuries involving both dermal and epidermal elements where the donor sites for autograft are very limited, the permanent wound closure is enabled by cell cultured skin substitutes (Stashak et al., 2004; Supp and Boyce, 2005; Balasubramani et al., 2001).

At present, there is no strict distinction between wound dressings and skin substitutes. If synthetic or biological polymers are used alone or associated to each other, they are termed as wound dressing. On the other hand, they are called as skin substitutes when synthetic or biological proteins are combined with matrix proteins and matrix proteins are cultured with cells. When matrices are seeded with cells they are more truly considered as cell cultured skin substitutes (Ramos-E-Silva and Castro, 2002).

Briefly, wound dressing is a biomaterial of conventional, synthetic or biological origin that is designed to recover the normal physiological and structural properties of the wounded skin.

1.2.1. History of Wound Dressings

The written records like Sumerian cuneiform tablets before 2000 BCE and Egyptian papyruses from 1550 and 1650 BCE provide specific details on how the mankind managed with wounds in the past. For instance, the former describes the application of poultices formed of mud, milk, and plants to wounds, and the latter provide specific details of how to wash the wound, prepare and apply plasters of honey, plant fibers, etc, and then bandage the wound. Since those times, even though the enhancement of wound dressing material from readily available materials in nature to materials specifically designed by man to provide particular benefits for wound healing has been very slow, the wound dressing materials has evolved significantly in the past quarter century (Ovington, 2007).

1.2.2. Properties of an Ideal Wound Dressing

Requirements for an ideal dressing are listed below:

- ✓ Protect the wound from secondary infections through bacterial invasions
- ✓ Prevent water loss
- ✓ Have controlled water vapor permeability (should prevent both desiccation and fluid accumulation at the wound site)
- ✓ Provide a moisturized wound healing environment
- ✓ Permit oxygen and carbon dioxide exchange
- ✓ Promote fluid drainage ability (absorb the toxic wound fluids and exudates)
- ✓ Drape well to surface irregularities of wound surface

- ✓ Rapid and sustained adherence to wound bed (if it is not biodegradable, removable without causing trauma to the wound)
- ✓ Have controlled degradation
- ✓ Reduce scar formation
- ✓ Protect the skin-underlying tissues (newly formed tissues) against mechanical damage (have enough tensile strength) and have acceptable handling qualities (able to resist tearing and disintegration when wet or dry)
- ✓ Elastic to permit motion of underlying tissue
- ✓ Non-antigenic and nontoxic
- ✓ Have minimal inflammatory or foreign body response
- ✓ Biocompatible
- ✓ Thermal insulation
- ✓ Sterilizable
- ✓ Stable during storage and have minimum storage requirements
- ✓ Able to act as drug delivery reservoir for any medication or growth factor
- ✓ Readily available in any size
- ✓ Improve the healing process by actively attracting the cells to the wound area
- ✓ Low cost
- ✓ Minimize nursing care of wound, patient discomfort
- ✓ Translucent properties to allow direct observation of healing

(Ehrenreich and Ruszczak, 2006; P.G. Shakespeare, 2005; Sheridan and Tompkins, 1999; Wang et al., 2006; Deng et al. 2007; Kokabi et al., 2007; Yang et al., 2007; Wittaya-areekul and Prahsarn, 2006; Adekogbe and Ghanem, 2005; Stashak et al., 2004; Ramos-E-Silva and Castro, 2002).

1.2.3. Classification of Dressings

Wound dressings may either be classified according to the duration of application or to the nature of its material (Sai and Babu, 2000; Sheridan and Tompkins, 1999) as follows:

According to the mode of application

1. Temporary: Temporary dressings protect the wound from mechanical trauma, form a physical barrier to bacteria and provide a water vapor transmission similar to skin for a specific period of time. It may be applied on superficial wounds to facilitate pain control and epithelialization and on full thickness wounds after excision while waiting autografting (Sherian and Tompkins, 1999).

2. Permanent: These dressings are left on the wound until complete healing is reached or until epidermal grafting. Autologous skin grafts are used as permanent wound closures. Also, various forms of collagen collagen based dressings have been used as permanent dressings (Silver, 1994; Lee et al. 2001; Balasubramani et al., 2001; Ramos-E-Silva and Castro, 2002). These were exemplified in the subsequent parts.

Based on the type of material used for the preparation of dressing

1. Conventional
2. Synthetic
3. Biological dressings

Since it is easier to make a classification based on the type of material used for the production of dressing they have been categorized accordingly in the following sections.

1.2.3.1. Conventional Dressings

The dressings made up of fabric material, such as gauze, provide little or no occlusion, allow excess moisture evaporation resulting in desiccating wound bed and cannot prevent entry of exogenous bacteria into the wound. Therefore, compound dressings in which wide mesh gauze is impregnated with medical grade paraffin or silicone were developed. Highly absorbent cotton gauze dressings facilitate the debridement of heavily contaminated exudative and necrotic wounds. The wound debris and necrotic tissues are removed as the dressing bounded to wound fibrin is peeled off. However, since corresponding deficiencies of conventional dressings restricted its application on full thickness wounds, more advanced dressing typessuch as synthetic and biological dressings have been developed (Stashak et al., 2004).

1.2.3.2. Synthetic Polymeric Dressings

Synthetic dressings based on polymeric sheets (Table 1.7) are only suitable for superficial wounds but not for deeper wounds. The polymeric sheet dressings were further improved by the production of composite dressings composed of two or more layers of polymers. The inner layer is designed to have maximum adherence and elasticity while the outer layer makes the dressing durable. Nevertheless, these dressings are used temporarily and do not help in extensive and deep burn injuries. Consequently, the limitations of these dressings have been endeavored to overcome with the search continuing to develop biological dressings with superior qualities and functions (Balasubramani et al., 2001).

Table 1.6. Summary of commonly used synthetic polymer based wound dressings (Balasubramani et al., 2001).

Type	Composition	Examples	Advantages/ disadvantages
Films	Polymer sheet+ adhesive; Polyurethane, Polyethylene, Polycaprolactone, Polytetraflouraethylene.	Tegaderm, Dermafilm, Opsite, Opraflex.	➢ well suited to superficial wounds ➢ lack of absorbing capacity ➢ impermeable to water vapor and gases
Foams and sprays	Sheets of foamed solutions of polymers; Polyvinylalcohol, Polyurethane.	Silastic foam, Lyofoam.	➢ provide thermal insulation ➢ maintain a moist environment at the surface of the wound ➢ gas permeable ➢ non adherent ➢ light ➢ comfortable

Table 1.6. (continued)

<u>Composite dressings</u> a. Hydrocolloid b. Hydrogel sheets c. Hydrogel amorphous d. Super absorbents	Laminates of 2 or more layers a. elastomeric adhesive+ gelling agents	Granuflex, Epigard, Biobrane	➢ adherence ➢ availability ➢ sterility ➢ long shelf life ➢ low cost
	b. 3D Networks of cross-linked hydrophilic polymers Polyethylene oxide, Polyacrylamide Polyvinylpyrolidine	Vigilon	➢ unique cooling ability (beneficial for thermal burns) ➢ slippery to use ➢ difficult to keep in place in high sheer stress
	c. Similar in composition to hydrogel sheets except that the polymer has not been cross-linked to form a sheet	IntraSite	➢ unique in their ability to donate moisture to a dry wound eschar ➢ facilitate autolytic debridement in wounds ➢ may be difficult to retain it in the wound bed owing to its viscosity
	d. An island configuration with an extra thin hydrocolloid as the adhesive portion	Combiderm Conva Tec	➢ used as temporary dressing ➢ does not help in massive burn injuries

1.2.3.3. Biological Dressings

Usually, skins from animal or human sources and various formulations-combinations of collagen, elastin and lipid are used as biological dressings. The superiorities of biological dressings on synthetic ones involve the followings;

1. Permit less painful dressing changes;
2. Facilitate debridement of wounds;
3. Increased capillary content of granulation tissue bed for autografting of deep wounds;
4. Decrease healing time of partial thickness burns and donor sites
5. Improve quality of healing, inhibit excessive fibroblasts and decrease contraction.

Biological dressings range from skin grafts (autograft, allograft from human and xenografts from other species) to films, sponges and composites of reconstituted collagen from bovine and other sources, and engineered skin substitutes (Sai and Babu, 2000; Silver , 1994).

1.2.3.3.1. Tissue Grafts

Skin grafts harvested from the patient's own skin (autograft) is used as the ultimate skin graft whenever it is plausible. The autografts are classified as split thickness skin grafts (STSG) or full thickness skin grafts (FTSG). These grafts contain the entire thickness of epidermis and variable thicknesses of the dermal component. If the entire thickness of dermis is included, it is termed FTSG, if less than the entire thickness is included then it is referred to STSG. FTSGs have higher incidence of failure, because their metabolic needs are higher than STSGs. On the other hand FTSGs have better cosmetic results. Both grafts leave a second wound and they are not likely to find in the desired size in case of extensive skin burns, so allografts are preferred. STSGs are accepted as the best material to use in surgical repair of burns (http://emedicine.medscape.com/article/876290-overview; Shakespeare, 2001).

Allograft skin can either be obtained from a family member or from cadavers. The latter is most common and if fresh frozen lyophilized allograft is used for thermal injuries, especially for extensive full thickness burns, it will be the most effective. Although amniotic membranes have also been used as allograft, they are not effective in the prevention of evaporative water loss and thus causing desiccation of the wound (Upjohn et al., 2004).

Grafts from animal sources have been investigated as an alternative to allograft. Among them, porcine skin is the most commonly used xenograft owing to structural similarity to human skin with respect to its texture, adherence and collagen content despite being dissimilar at the microscopic level.

Major disadvantages of allografts/xenografts are:

- Troublesome and costly to obtain
- Limited supply
- Variable in quality
- Difficult to sterilize and store aseptically
- Have a short shelf-life
- Potentially antigenic
- Carry a risk of unusual contaminants and viruses
- May provide hypersensitivity to one or more complex constituents

Owing to these disadvantages of allografts/xenografts, collagen dressings and tissue engineered skin substitutes made up of skin matrix components (collagen) with or without cells were developed. (Sai and Babu, 2000; Singh et al., 2006; Metcalfe et al., 2007)

1.2.3.3.2. Reconstituted Collagen Dressings

Various forms of collagen based materials were investigated for wound dressing applications.

Collagen films are made by casting collagen solutions onto non-sticky surfaces. These films cross-linked chemically or physically elicit a very mild inflammatory response and allow the adherence of fibroblast to collagen surface. Since these films are very thin, they are difficult to handle (Lee et al., 2001). Collagen was combined with other materials to enhance its healing effect. Collagen films incorporated with antioxidants were developed to heal the dermal wounds (Gomathi et al., 2003).Collagen was also combined with other proteins such as chitosan to test the in vitro culture of human epidermoid carcinoma cells (Shanmugasundaram, 2001). Composite collagen/polycaprolactone films produced by Dai et al. (2004) were favourable substrates for growth of fibroblasts and kerationocytes and might be suitable for skin repair.

Collagen sponges are prepared by freeze-drying a collagen solution to get a porous structure. Collagen sponges contain large pores and channels within which cells can migrate and grow. These dressings have been utilized as both temporary and permanent coverings for dermal defects in animals and humans. Collagen sponges are able to easily absorb large quantities of tissue exudate, adhere smoothly to the wet wound bed with preservation of low moist climate as well as its shielding against mechanical harm and secondary bacterial infection (Lee et al., 2001). Ma et al. (2003) suggests that collagen/chitosan sponges cross-linked by GTA are potential candidates for dermal equivalent with enhanced biostability and good biocompatibility. Sheu et al. (2001) investigated the influence of GTA on collagen matrices and a GTA concentration of 0.12% seemed to be optimal in terms of contraction and cell cytotoxicity.

Electrospun nanofibrous membranes are fabricated by an electrospinning process for which a polymer solution placed inside a syringe is driven out from a metal capillary that is connected to high voltage power supply. Nanofibers are then collected in the form of a non-woven mat on a grounded collector after solvent evaporation. Chen et

al. (2007) claimed that wound dressings made by electrospinning process might potentially be advantageous over conventional processes. Therefore, Chen and co-workers successfully produced an electrospun nanofibrous membrane and investigated its effect on wound healing. In another study, Rho et al. (2006) indicated that electrospinnig of type I collagen produces a nanofibrous matrix that may be good candidate for wound dressing applications. Powell et al. (2008) compared electrospun and freeze-dried collagen scaffold and found that the former could be advantageous for its optimal cellular organization and potential reduction in wound contraction.

1.2.3.3.3. Tissue Engineered Skin Substitutes

Tissue engineered skin substitutes could either be designed as a matrix system inoculated with cells from which skin substitutes are created or as a cell-free matrix material that induces specific cell reactions with the host tissue, thereby allowing in vivo formation of tissue regeneration and improvement of nerve regeneration and blood vessel formation (Ramos-E-Silva and Castro, 2002; Kearney, 2001). There are several skin substitutes available on the market (Table 1.8).

1.2.3.3.3.1. Acellular Skin Substitutes

Neonatal (allogeneic) fibroblasts cultured on nylon fibers embedded into a silicone layer for 4 to 6 weeks forms a dense cellular matrix containing high levels of secreted human matrix proteins with several growth factors (TransCyte^{TM}). The fibroblasts become nonviable after freezing.

A skin substitute composing of skin extracellular materials, collagen and chondroitin-6-sulfate as the dermal substitute and a disposable silicone sheet as the artificial epidermis without any cell culturing (Integra^{TM}) is designed to constitute the epidermal and dermal layers of normal skin. The porous matrix structure can be controlled by freeze drying process and the vascularized structure formed is suitable

for cell ingrowth. The silicone epidermis layer is removed before the application of split thickness epidermal autograft or autologous cell cultured skin substitute.

Porcine collagen chemically cross-linked with an aldehyde is used as a temporary skin substitute (E-Z-Derm^{TM}). It acts as an acellular dermal matrix.

Biobrane^{TM} consists of an outer silicone membrane with a nylon fabric partially imbedded into the layer. Porcine collagen is incorporated into the silicone and nylon components of the film by chemical cross-linking.

De-epidermized acellular graft obtained from porcine source (Xenoderm^{TM}) or human cadaver (Alloderm^{TM}) is commercially available.
Another skin substitute application of porcine source (Oasis^{TM}) consists of small intestinal submucose. The matrix contains collagen type I, II, V and growth factors (Balasubramani et al., 2001; Ramos-E-Silva and Castro, 2002; Boyce S.T., 2001; Shakespeare, 2005).

1.2.3.3.3.2. Cell Cultured Skin Substitutes

1.2.3.3.3.2.1 Epidermal Skin Substitutes

One of the oldest methods (Epicel^{TM}, commercially available in 1988) is based on culturing autologous keratinocytes on a feeder layer of lethally irradiated 3T3 mouse fibroblasts. This technique was not only used in extensive burn wounds (over 95% body surface area) and proved to be life saving, but also found to be beneficial for acute and chronic wounds. The cultured epidermal autograft is used permanently owing to the elimination of rejection problem. Due to the time required for their preparation and the need for biopsy, allogeneic keratinocyte (neonatal foreskin) sheets are preferred to accelerate the preparation. In spite of being a temporary wound covering, it promotes permanent re-epithelialization by host kerationocytes.
Since extremely delicate sheets yield poor cosmetic results and blistering of the epidermis, a cell growth and delivery substrate was used. A product available in the UK, Germany and Italy (VivoDerm) was produced by seeding hyaluronic acid

membrane having laser-drilled microperforations with autologous kerationocytes (Ramos-E-Silva and Castro, 2002; Jones et al., 2002).

1.2.3.3.3.2.2. Dermal/Epidermal Composite Skin Substitutes

The success rate of epidermal grafting depends on the attachment of the keratinocytes to the wound bed, more specifically to basement membrane. However, in full thickness burns where epidermal and dermal layers are annihilated completely replacement of dermis is logical to create an attachment site for the subsequent autologous/allogeneic kerationocyte grafting. Also, to replace the dermis layer is very crucial to prevent excessive formation of granulation tissue which gives rise to scar. Therefore, several skin substitutes consisting of dermal and epidermal layers were produced.

Human neonatal fibroblasts (allogeneic) cultured on a bioabsorbable net with circulating nutrients attach, multiply and begin secreting collagens and growth factors and a solid tissue consisting of collagen proteins and cells is formed (Dermagraft^{TM}). The net degrades after 3-4 weeks and the fibroblast collagen matrix is used alone or with possible epidermal cultures.

Apligraf^{TM} consists of both dermal and epidermal layers. The dermal equivalent matrix made up of type I bovine collagen with human dermal fibroblast (allogeneic), and the overlying epidermal layer cultured with human keratinocytes resembles human skin histologically and produces a great number of cytokines and growth factors.

A composite skin equivalent (OrCel^{TM}) consisting of a cross-linked bovine collagen sponge and a nonporous collagen gel is seeded with fibroblasts (allogeneic) on the porous side and with keratinocytes (allogeneic) on the other side. Culturing continues for 10 to 15 days (Ramos-E-Silva and Castro, 2002; Balasubramani et al., 2001; Kearney, 2001; Boyce, 2001).

Table 1.7. Skin substitutes present at the market (Lee, 2000; Ramos-e-Silva et al., 2002; Jones et al., 2002).

Product Name	Advantages	Disadvantages
Epicel	Coverage of large areas Permanent wound coverage Minimal risk of disease transmission	2-3 weeks intervals for culture Fragile, difficult to handle
Alloderm	Immediate availability Immunologically inert	Preservation Infection transmission risk Two surgical procedures
Integra	Immediate wound coverage Reduces the thickness of subsequent skin graft Reduces contracture No inflammatory response	Complete wound excision Two steps procedure Susceptible to infection Its cost Fluid collection underneath
Dermagraft	Immediate availability Good resistance to tearing Easy to handle No rejection	Expensive
Apligraf	Immediate availability Avoidance of donor site wound and surgical procedure Easy handling	Limited viability High cost Short shelf life (only 5 days)
OrCel	Immediate availability	Little clinical data to support its use
Oasis	Immediate availability Long shelf life	Little clinical data
E-Z-Derm	Immediate availability Long shelf life	Little clinical data Possibility of disease transmission Increased amount of exudates

1.3. General Properties of Materials Used in the Study

Proteins are essentially composed of amino acids and they construct the basis for structural or mechanical component of many tissues. Furthermore, they play an important role as substrates for cell adhesion and migration, as signaling molecules, enzymes, and/or active regulators of cellular differentiation, functionality etc. Natural materials and industrially created polymers are used as biomaterials for tissue repair and replacement. Examples of natural materials include polypeptides, GAG's, fibronectin, collagen, hydroxyapatites, chitosan, and alginates. Most common examples of synthetic materials include polyglycolide acid (PGA), polylactic acid (PLA), polytetrafluoroethylene (PTFE), polycaprolactone (PCL) and so on. The former is superior to the latter in that they are expected to undergo naturally controlled degradation processes releasing less toxic products and cause to low chronic inflammatory response (Guelcher and Hollinger, 2006; Metcalfe, 2007).

1.3.1. Collagen

1.3.1.1. Sources and Refinement

Collagen is the primary structural material of vertebrates and is the most abundant mammalian protein accounting for about 20–30% of total body proteins (Harkness, 1961). It is present in tissues of primarily mechanical function. About one half of the total body collagen is in the skin and about 70% of the dermis of skin and tendon is collagen. A variety of cells, perhaps most notably fibroblasts, produce collagen. For the purposes of engineering tissues and implants, collagen is often harvested from bovine, equine, and porcine tissues, and can be processed into various forms including films, mats, fibers, and gels. In order to avoid rapid degradation in vivo and to increase mechanical strength, collagen must be fixed, or cross-linked, either chemically or physically. Although physical cross-linking methods has the advantage of not leaving toxic chemical residue, chemical fixatives are more commonly used (Guelcher and Hollinger, 2006; Lee et al., 2001).

1.3.1.2. Molecular Structure

The primary structure of collagens can be represented by the motif of Glycine-Proline-Hydroxyproline-Glycine-X-Y where X and Y can be any amino acids. This sequence forms an alpha chain and every collagen molecule contains a triple α-helix, which is the molecular basis of tropocollagen, precursor of collagen. According to the type of tissues the individual α-chains differ. For instance, type I collagen consists of three coiled subunits composed of two α-1 (I) chains and one α-2 (I) chain. Each α chain winds around each other to form a right-handed triple helix. The three chains are held together strongly by H bonds between glycine residues and between the hydroxyl (-OH) groups of hydroxyprolines. In addition, there are cross-linkages via lysine among α helixes. Hence, the amino acid composition of a collagen species also affects cross-linking and thereby mechanical properties. Lysine and hydroxylysine are necessary for natural intra- and inter-molecular cross-linking of collagen (Angele et al., 2004). Therefore, the more lysine and hydroxylysine present in a collagen molecule, the more resistant it will be to enzymatic and thermal degradation

As hydroxyproline is present in minute amounts in other proteins, it is considered as unique to collagen; therefore, assays of hydroxyproline are frequently used to determine the collagen content in a given tissue. Propeptide regions, which are thought to play a role in the initial assembly of the triple helix, are present at the ends of procollagen molecules (Figure 1.4.A). After secretion from cells, the propeptides are enzymatically cleaved from the procollagen molecules to form collagen molecules with 1.5 nm diameter and about 300 nm length (Figure 1.4.B). Collagen molecules undergo a self assembly process to form collagen fibrils (10-300 nm in diameter). This assembly results in staggered array of collagen molecules, in which overlapping rows of collagen molecules are staggered with respect to one another by a distance of 64 to 67 nm forming a banded structure (Figure 1.4.C) (Dee et al, 2002; Park and Bronzino, 2003; Meyers et al., 2008).

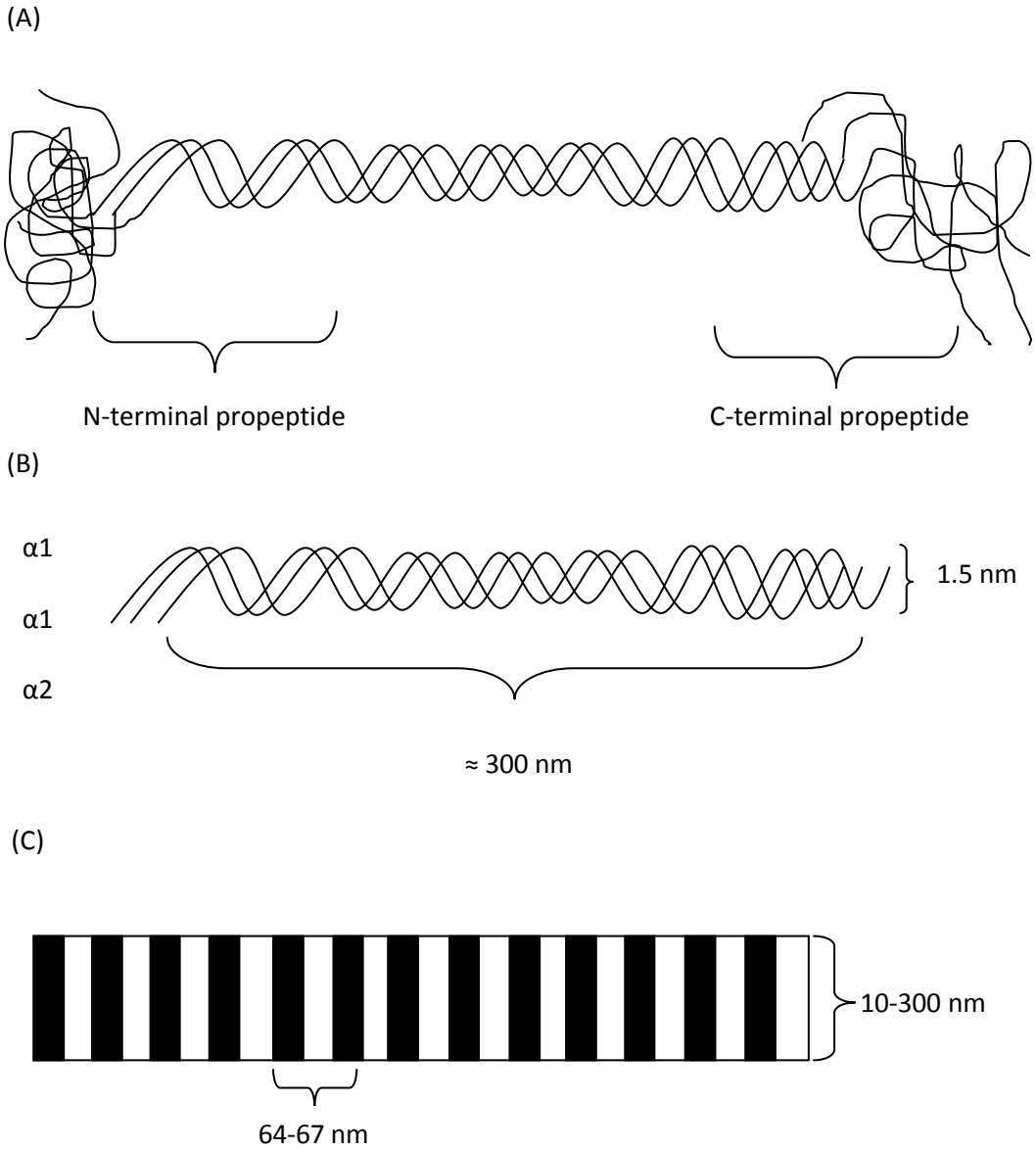


Figure 1.4. Collagen Structure. (A) Procollagen molecule. (B) Collagen molecule. (C) Banded structure of collagen fibrils. (Adapted from Dee et al., 2002)

1.3.1.3. Types and Distributions

Over 20 types of collagen have been identified. Type I collagen is distributed in most tissues and comprises the largest proportion of the total collagen within the human body (Fung, 1993). Type I collagen is found mainly in bone, tendons, ligaments, and skin (Guelcher and Hollinger, 2006).

1.3.1.4. Mechanical Properties and Stability of Collagen

With over 20 species of collagen, a wide spectrum of chemical and mechanical properties could be attained as all provide different characteristics. Yet, some of the fundamental mechanical properties (and structure-property relationships) of collagen needs to be fully explored. Collagen being the basic structural component in our body plays the same role as steel does in structures.

In general, temperature and pH are known to affect protein stability. For collagen, the material behavior is relatively unaffected over the range of temperature from 0 to 37 ^{o}C (Rigby et al., 1958). However, exposing collagen to temperatures above 37 ^{o}C or to a low pH solvent causes the denaturing of its triple-helix structure (Yannas, 1996; Mirnajafi et al., 2005).

Nonlinear stress-strain curve obtained from mechanical tests of collagen fibers and soft collagenous tissues is displayed in Figure 1.5. This nonlinearity is a result of the natural "crimp" in relaxed collagen. As the relaxed collagen fibers continue expanding under low strains, the collagen molecule starts becoming more linear. The part of the curve below the linear region is known as the "toe region". After this region, the stress-strain curve for collagen reaches to a generally linear region similar to that demonstrated by a linear elastic material. Finally, the collagen will undergo bond stretching and eventually break (Guelcher and Hollinger, 2006; Meyers et al., 2008).

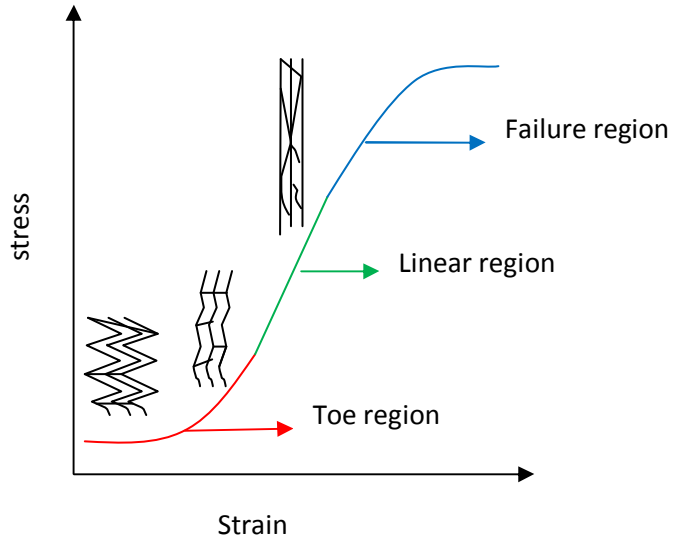


Figure 1.5. Characteristic stress strain curve of collagen and the location of the toe region (Adapted from Guelcher and Hollinger, 2006)

The tensile modulus (obtained from the linear portion of the stress-strain curve) for collagen fibers has a reported range between 350 and 1000 MPa (Fung, 1993; Gentlemen et al., 2003). The tensile strengths for collagen fibers were shown to range from 50 to 100 MPa (Fung, 1993). When compared to large diameter fibers, smaller diameter collagen fibers tend to display larger values of modulus and peak stress. It has been suggested that larger-diameter fibers are more likely to possess defects and thus break more easily. Besides that smaller fibers have a larger surface-to-volume ratio and allow greater number of cross-links per fiber unit length, which would make the fiber stiffer (Gentleman et al., 2003). The tangent modulus is a material property and therefore should be independent of the size of the specimen. However, different lengths of collagen fibers, either fabricated in the same way or dissected from the same tissue, have been shown to have significantly different moduli (Haut, 1986; Gentlemen et al., 2003).

1.3.1.5. Physiological Responses

One benefit of biomaterials, like collagen, is that the materials are normally known by the living system. Collagenases and metalloproteinases are produced by cells to degrade collagen in tissues. The products of this degradation will be amino acids that the body copes with in the course of its normal metabolic processes.

The interaction of collagen with the surrounding environment is regulated with its quaternary structure. For example, platelet aggregation has been shown to be a property of the quaternary structure (Yannas, 1996). Collagen acts as chemo-attractant to leukocytes and fibrogenic cells (Gentlemen et al., 2003) and binds to growth factors and cytokines (Gelse et al., 2003).

The triple helix structure of collagen is present in many species. Especially, type I collagen is abundant in most organisms, and therefore a better toleration across species and a lower immune response than other collagen types are expected (Yannas, 1996). Patients awaiting an implant were tested to determine whether they have a pre-existing allergy to collagen and it was reported that only up to 4 % of a population had a pre-existing allergy to bovine collagen (Cukier et al., 1993; Soo et al., 1993; Cretel et al., 2001). The adverse reactions to implanted collagen in patients without preexisting allergies were also found to be rare (< 3%) (Lynn et al., 2004).

1.3.1.6. Biomedical Applications of Collagen

Since collagen is found in nearly every tissue in the human body and easy to process in the laboratory it has been investigated broadly for biomedical applications. Its excellent biocompatibility and biodegradability render collagen a useful candidate for this purpose. In addition to being used for various drug delivery systems it was also used for tissue engineering applications including skin replacement, bone substitutes and artificial blood vessels and valves, tendon and ligament, peripheral nervous systems, cartilage and meniscal tissue (Lee et al., 2001; Guelcher and Hollinger, 2006).

1.3.2. Sericin

1.3.2.1. Sources and Refinement

Silk derived from silkworm Bombyx mori is mainly composed of sericin and fibroin proteins. Sericin constituting 25–30 % of silk envelops the fibroin fiber with successive sticky layers that help in the formation of a cocoon. It is estimated that 1 million tons of cocoons (fresh weight) is produced worldwide and sericin is discarded as a waste product. Although at present, sericin is mostly discarded in silk processing wastewater, the recovery and recycling of this sericin could provide a significant economic and social benefit because depletion of 50,000 tons of sericin from 400,000 tons of dry cocoon is a great loss.

Sericin is a water soluble protein. When dissolved in a polar solvent, hydrolyzed in acid or alkaline solutions, or degraded by a protease, different molecular size distributions ($\leq$20 kDa or $\geq$20 kDa) of the resulting sericin molecules occur depending on factors such as temperature, pH, and the processing time.

The small sericin peptides are soluble in cold water and can be recovered at early stages of raw silk production while the larger sericin peptides are soluble in hot water and can be obtained at the latter stages of silk processing or from silk degumming processes (Zhang, 2002).

1.3.2.2. Molecular Structure

Sericin having a wide molecular weight range of about 10 to over 300 kDa is a macromolecular protein. It is made of 18 amino acids most of which have strongly polar side groups such as hydroxyl, carboxyl, and amino groups. In addition, the amino acids serine and aspartic acid constitute approximately 33.4 % and 16.7 % of sericin, respectively. Serine is thought to make sericin an excellent moisturizing agent (Zhang, 2002; Kundu et al., 2008).

1.3.2.3. Physiological Responses

The silk was known to cause allergic reactions (Hollander, 1994; Zaoming et al., 1996; When et al., 1990; Kurosaki et al., 1999). The glue-like protein sericin has been implicated as the causative agent for immune responses to silk, but only when it is found in conjuction with fibroin. Sericin by itself did not elicit an immune response (Panilaitis et al., 2003). Meinel et al. (2005) showed that the inflammatory tissue reaction and foreign body response to silk films was similar or less than collagen films.

1.3.2.4. Biomedical Applications of Sericin

Various biomedical application areas of sericin were reviewed by Zhang et al. (2002) and Kundu et al. (2008). The effect of sericin on the attachment of primary cultured human fibroblasts was studied by Tsubouchi et al. (2005). It was found that the attachment of these cells was enhanced with sericin. The studies conducted by Aramwit and Sangcakul (2007) about the wound healing effect of sericin cream in rats suggested that sericin had wound healing effects without causing allergic reactions. Tsubouchi (1999) developed a fibroin-mixed-sericin wound dressing that could accelerate healing and could be peeled off without disturbing the newly formed skin. The antioxidant action of sericin was shown by Kato et al. (1998). Antioxidant potential and photoprotective effect of silk protein sericin against hydrogen peroxide-induced oxidative stress in skin fibroblasts and skin keratinocytes was studied by Dash et al. (2007 and 2008). Minoura et al. (1995) and Tsukada et al. (1999) investigated the attachment and growth of animal cells on films made of sericin and fibroin. Films of pure component proteins (i.e., fibroin or sericin) permitted cell attachment and growth comparable to that on collagen, a widely used substrate for mammalian cell culture. In another study made by Murase (1994), films made of sericin and fibroin were found to have excellent oxygen permeability and showed high similarity to human cornea in its functional properties. Hence, it was thought as promising to form artificial corneas. A novel mucoadhesive polymer has been

prepared by template polymerization of acrylic acid in the presence of silk sericin (Ahn et al., 2001). It was also shown by Tamada (1997) that biomaterials with anticoagulant properties could be prepared by sulfonation treatment of sericin and fibroin proteins. The Anticoagulant activity of sulfated sericin was estimated at 1/10 to 1/20 of heparin by Tamada et al. (2004). The findings of Zhaorigetu et al. (2003) suggest that sericin has suppressing activity against both chemical and UV radiation induced skin tumorigenesis by reducing oxidative stress. A sericin diet is reported to suppress the incidence and number of colon tumors (Sasaki, 2000; Zhaorigetu et al, 2001).

1.4. Aim of the Study

Collagen based matrices have been used for many biomedical applications successfully as previously stated in the corresponding parts. These matrices could be assumed to be very promising in wound dressing applications since collagen is known to be involved actively in wound healing process. In addition to being chemoattractant to cellular elements of healing such as granulocytes, macrophages and fibroblasts it promotes wound maturation by providing a scaffold that is able to transit more quickly to the mature collagen production and alignment phases of healing, and also provides a template for cellular attachment, migration and proliferation (Sai and Babu, 2000). In this study, collagen was used to benefit these properties. However, the novel part of the present study was the investigation of the sericin as a wound dressing material since there are not many studies on the effect of sericin in wound dressing applications. At present, sericin is mostly discarded in silk processing wastewater, but the recovery and recycling of this sericin could provide a significant economic and medical benefit because depletion of 50,000 tons of sericin from 400,000 tons of dry cocoon is a great loss (Zhang, 2002). In addition, sericin is associated with very important attributes such as excellent oxygen permeability, antioxidant action, moisture regulating ability, antibacterial ability, UV resistance, anticancer and anticoagulant property (Kato et al., 1998; Murase, 1994; Zhang et al., 2002; Cho et al., 2003; Tamada, 1997; Zhaorigetu, 2003; Mondal et al., 2007).

Sericin with the above mentioned properties is likely to be a highly suitable substance for the development of wound dressing biomaterial and needs more detailed characterization for wound healing purposes especially in its *in vitro* efficacy for skin tissue engineering. Accordingly, all of these attributes could be exploited by producing a composite sericin and collagen wound dressing. To produce a composite membrane, sericin and collagen solutions were mixed at different weight ratios and simply casted on smooth surfaces and air dried. These membranes were also cross-linked with GTA solution to enhance the material properties such as mechanical properties and resistance to degradation. For *in situ* characterization of membranes, degradation of different membrane groups with and without cross-linking was studied in aqueous environment. Then, the most stable membrane groups were selected as the standardized test groups. Further characterization tests were also conducted to understand the water absorption, water vapor penetration, oxygen permeability, microbial penetration and mechanical properties of these standardized membrane groups. For visual examination of the membranes, SEM and light microscopy analysis were applied. For *in vitro* biocompatibility and bioactivity studies, two different cell lines, human HaCat keratinocytes and mouse 3T3 fibroblasts were used. In these studies, keratinocytes and fibroblasts were seeded on membranes and the cells were incubated for 1 week. After 1, 4 and 7-day incubation periods, MTT cell viability tests were performed to assess proliferation. In addition, cell attachment, morphology and spreading-distribution were scrutinized with light microscopy and SEM.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

For membrane preparation: Commercial sericin was purcahsed from a Brazillian company and collagen (Gelfix Lyophilized Type I Collagen pad) was obtained from Isse International Eurosearch S.r.l., Milano Italy. Acetic acid (100%) was obtained from Riedel-deHaën, Germany. Glutaraldehyde solution (50%) was obtained from Sigma-Aldrich Chemie GmbH, Germany.

For in situ characterization experiments: Magnesium chloride hexahydrate ($MgCl_2.6H_2O$, extra pure), sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$), manganese (II) sulfate monohydrate ($MnSO_4.H_2O$), sulfuric acid (H_2SO_4, 95-98%), starch (soluble) and nutrient broth (for microbiology) were purchased from Merck, Germany. Sodium hydroxide pellets (NaOH, puriss) were obtained from Riedel-deHaën. Sodium iodide (NaI, $\geq$ 99%) was obtained from Sigma-Aldrich Chemie GmbH, Germany.

For in vitro studies: Foetal bovine serum (Gold), Dulbeco's Modified Eagle's Medium (DMEM) low glucose (1g/l) with L-glutamine, DMEM/ Ham's F-12 with L-Glutamine, trypsin-EDTA (10x) (0.5%/0.2% in D-PBS), penicillin/streptomycin (100x) were purchased from PAA Laboratories, Austria. Dulbecco's MEM (1x) (w D-Glucose (4.5g/l), w $NaHCO_3$ (3.7 g/l), w stable glutamine, w Na-Pyruvate) and trypsin-EDTA solution (0.25%/0.02% w/v in PBS w/o Ca^{+2}, Mg^{+2}) were obtained from Biochrom AG, Germany. Ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA, 99%), thiazolyl blue tetra-zolium bromide (MTT bromide, approx. 98 % TLC), trypan blue solution (0.4%) were purchased from Sigma-Aldrich Chemie GmbH, Germany. Dimethyl sulfoxide (DMSO, cell culture grade, min. 99.5

%) was obtained from AppliChem, Germany. Ethanol (absolute extra pure) waspurchased from Merck, Germany. Glycine (electrophoresis purity reagent) was obtained from Bio-Rad Laboratories, USA.

2.2. METHODS

2.2.1. Preparation of Sericin-Collagen Membranes with Optimization of Experimental Conditions and Materials

The experiments for the preparation of sericin/collagen membranes as wound dressing were started in the Biomaterials-Biomechanics laboratories of METU Engineering Sciences Department. The initial optimization studies of membrane preparation were carried in the Membrane Production Laboratory of London Imperial College, Chemical Engineering and Chemical Technologies Department in the framework of Scientific Partnership Program of TUBITAK and British Council. Similar conditions were established at METU and optimization tests were conducted on the first set of membranes (Table 2.1) to choose groups appropriate for wound dressing applications. Consequently, a second set of membranes (Table 2.1) from the standardized groups was prepared and tested further.

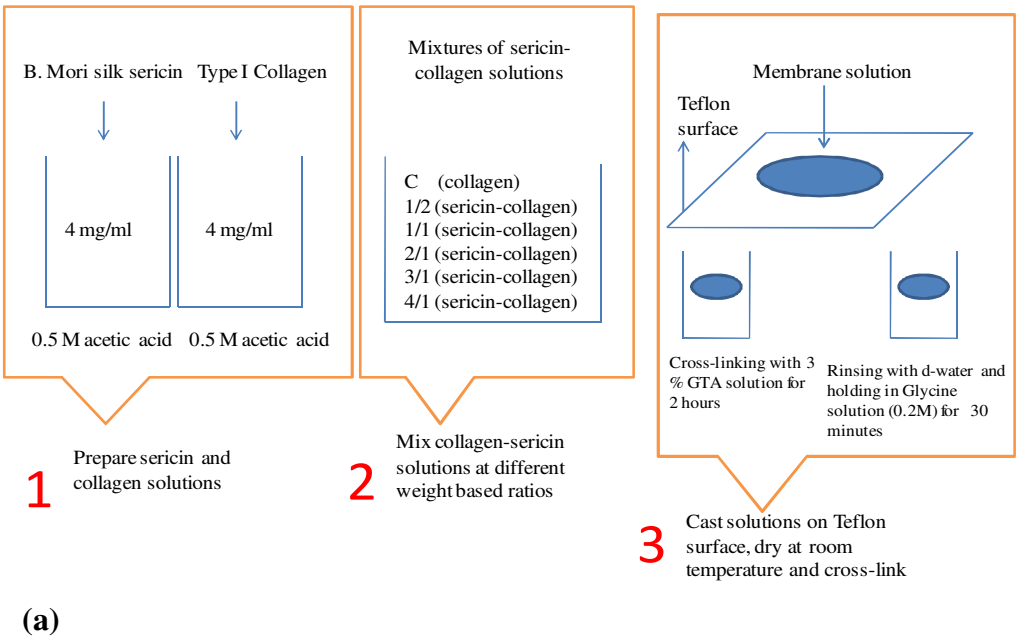
Sericin and collagen were both dissolved in 0.5 M acetic acid solvent to get a concentration of 4 mg/ml. Then the first set of sericin/collagen composite membranes was prepared by casting the mixtures of these solutions onto Teflon surfaces and air drying them at room temperature. To understand the effect of cross-linking treatment on the properties of membranes they were cross-linked with 3 % (w/v) glutaraldehyde (GTA) solution for 2 hours (Wittaya-areekul and Prahsarn, 2006) and GTA residues were rinsed with de-ionized water for 30 minutes (Figure 2.1a). Before the *in vitro* cytotoxicity tests the samples were also held in Glycine solution (0.2 M) for 30 minutes (Chen et al., 2008) to block the non-reacted aldeyhyde groups. After standardizing the membrane groups a second set of membranes consisting of thin and thick ones were prepared. Due to the reason that

thicker membranes are desirable for enhanced properties, somewhat thicker membranes were also produced in glass Petri plates by casting method (Figure 2.1.b). For thin membranes, the total weight of protein poured on Teflon surface was kept constant; on the other hand, the total weight of protein for thick membrane groups changed directly proportional with the ratio of sericin and collagen indicated in Table 2.1. All the membranes for the second set were also cross-linked with 3 % (w/v) GTA.

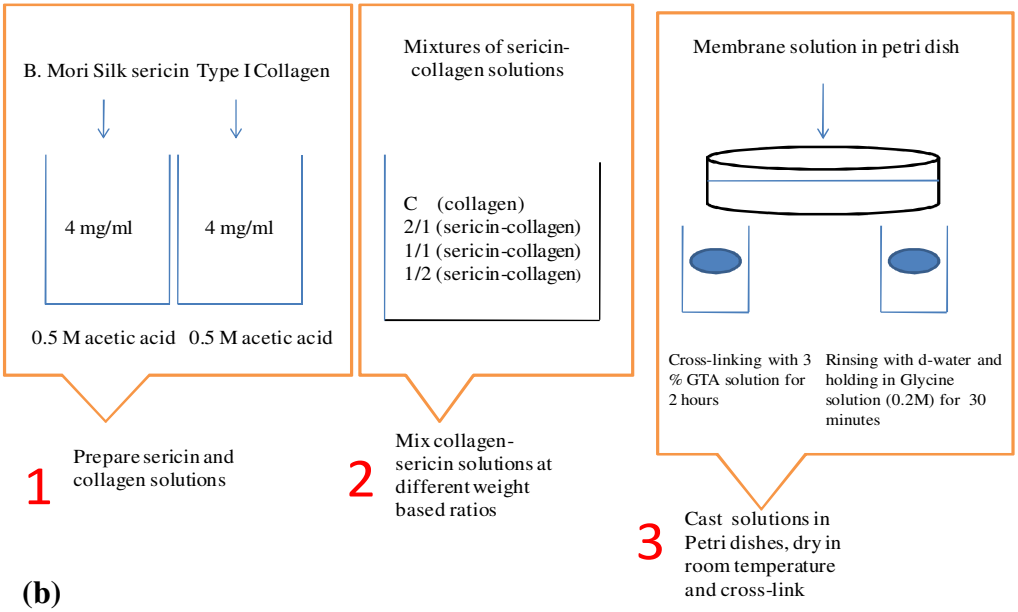
Table 2.1. Prepared sericin-collagen membrane groups

Group Names (Sericin-Collagen weight ratios)	Weight of Sericin (mg)	Weight of Collagen (mg)	Cross Linking Treatment
2/1i	24	12	-
3/1i	27	9	-
4/1i	28.8	7.2	-
Ci-x	-	36	+
1/1i-x	18	18	+
2/1i-x	24	12	+
1/2i-x	12	24	+
Ck-x	-	480	+
1/1k-x	120	120	+
2/1k-x	240	120	+
1/2k-x	120	240	+

The letter “i” represents thin membranes, whereas “k” is used for thicker ones. Membranes prepared from pure collagen solutions were indicated with “C”. Membranes treated with cross-linker were shown with “x”.



(a)



(b)

Figure 2.1. Preparation of (a) thin and (b) thick sericin/collagen membranes with solvent casting method

2.2.2. In Situ Characterization Tests

Before in situ tests, the thicknesses of sericin-collagen membranes were measured with micrometer (Vernier caliper). Their general microscopic structures were observed with light microscopy (Nikon Eclipse TS100, China) to see if there were any inhomogeneities or micro-scale defects within the membranes. For other in situ characterizations, water absorption, stability-degradation, microbial and water vapor penetration, oxygen permeability and mechanical tests were done.

2.2.2.1. Membrane Water Absorption Tests

A general method was used to measure the water absorption of membranes (Tanodekaew et al., 2004; Wittaya-areekul and Prahsarn, 2006; Kokabi et al., 2007). Membranes from each group (given in Tables 2.1) were cut into 2x2 cm^2 pieces and their dry weights (W_d) were measured with electronic balance. Each membrane was placed in plastic bottles (2.8 and 4 cm in diameter and height, respectively) immersed in de-ionized water and incubated at 37 ºC in an oven. The weight of each bottle was measured previously. All bottles were capped tightly to prevent any evaporative water loss. At different time intervals water inside the bottles were removed and excess surface water on swollen membranes was gently blotted with filter paper. The weights of these membranes were measured together with empty bottles and swollen membrane weights (W_s) were measured by taring. Equilibrium degree of swelling (EDS) was calculated using the following equation:

$$\text{EDS} = \frac{W_s - W_d}{W_d}$$

Accordingly, the water uptake values were normalized with dry membrane weights. The schematic representation of water uptake studies is shown in Figure 2.2. All tests were repeated 4 times with each membrane type and all measurements for the first and second set of membranes were done weekly lasting for 8 weeks and 4 weeks, respectively.

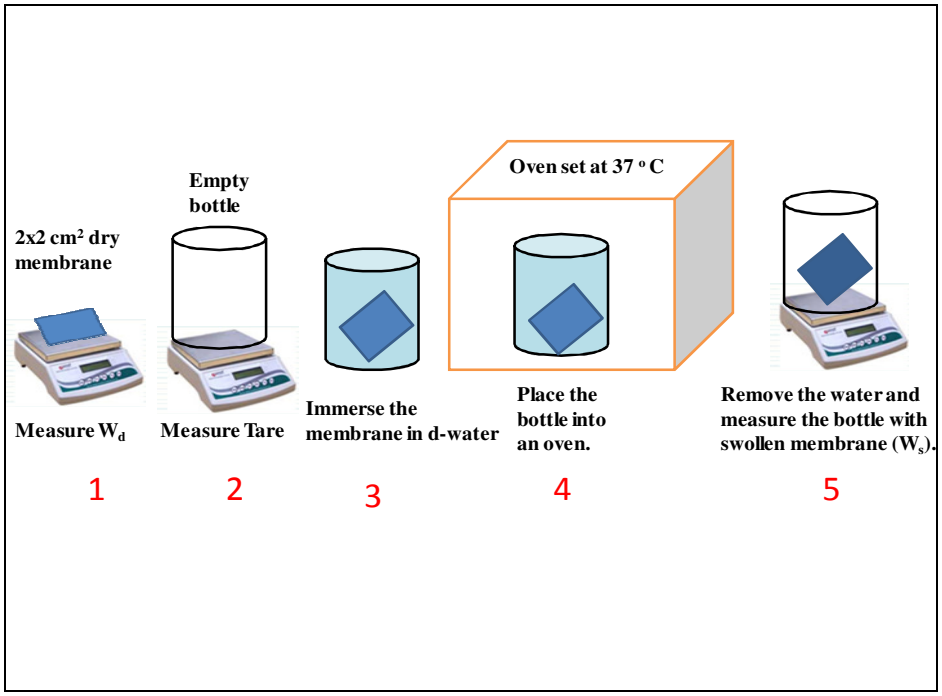


Figure 2.2. Experimental setup for testing water absorption of membranes

2.2.2.2. In Situ Degradation

The thin and thick membranes were first immersed in d-water containing bottles, then placed in an oven set at 37 °C and exposed to hydrolytic degradation for 8 and 4 weeks, respectively. For the optimization of the first set of membranes, the test was continued for 8 weeks. After the incubation period, the membranes were drained first and then left to dry out completely at 37 °C overnight. Their weights were then measured to calculate the weight loss. The structural integrity of membranes was also assessed visually after incubations.

2.2.2.3. Water Vapor Transmission Rate

Water vapor transmission rates (WVTR) were determined according to the ASTM method E96-90, Procedure D. An evaporimeter was constructed in a closed chamber to prevent variations owing to ambient conditions. Briefly, the system consists of a plastic box with an air tight cover and isothermal ambience at 35 °C, a digital

hygrometer with a continuous percent relative humidity (RH) and temperature display, and a reservoir of a saturated magnesium chloride solution to maintain the relative humidity at 40±2 % after equilibration. A cylindrical, plastic permeability cup filled with 20 g of de-ionized water and sealed with the test membrane at the top was placed inside the system (Figure 2.3). Evaporation of water through the test membrane was monitored by measuring weight change in plastic cup as an indication of daily loss of water. Then, the water vapor transmission rate (WVTR) was found by dividing the daily loss of water with the evaporation area of the permeability cup. (Mi et al., 2003; Wu et al., 1995; Cardona et al., 1996) The water vapor evaporation studies were carried out at least quadruplets (n≥ 4).

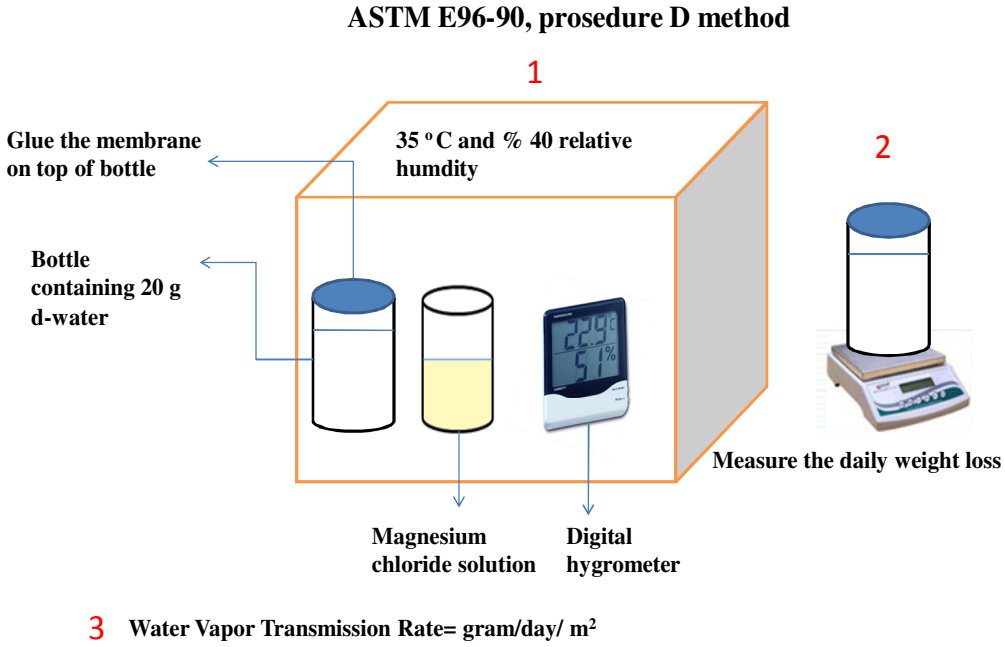


Figure 2.3. Experimental set up for testing water vapor transmission rate through membranes.

2.2.2.4. Oxygen Permeability Test

Studies for oxygen penetration through membranes were performed by attaching the membranes to the top of open Erlenmeyer flasks filled with 300 ml de-ionized water (Figure 2.4) (a modified method of Wittaya-areekul and Prahsarn, 2006). The negative control was the flask closed with an airtight cap while the positive control was the open flask allowing oxygen to enter into the flask and dissolve in the water. The test flasks were placed in an open environment under constant agitation with magnetic stirrers for 24 h. The collected water samples were then analyzed for dissolved oxygen content according to Winkler's method (Glazer et al., 2004). The test procedure followed was adopted from Standard Methods for the Examination of Water and Wastewater (1995, New York, American Public Health Association). In the Winkler's method, 1 ml of divalent manganese solution ($MnSO_4.H_2O$, 0.364 g/ml in d-water) is added into the test solution, followed by 1 ml of strong alkali-iodide solution (NaOH+NaI, 0.5 g/ml+0.135 g/ml in d-water). Under such conditions, any dissolved manganese (II) ion in water combines with hydroxide ions and $Mn(OH)_{2(s)}$ forms. Under the presence of a strong alkali, any dissolved oxygen in water oxidizes this manganese complex to Manganese (III), to ($Mn(OH)_{3(s)}$ .At that point of the experiment, $Mn(OH)_3$ causes to the formation of a brown precipitate at the bottom of the flask. The prevailing iodide (I) in water react with the precipitate after addition of concentrated sulphuric acid (1ml), producing Mn^{2+} and iodine (I_2) at the end. It is possible to correlate the free iodine (I_2) in reaction steps (Figure 2.5) to the original dissolved oxygen in the tested water. So, as shown in Figure 5, free iodine is produced at a rate of one I_2 molecule for each atom of oxygen (O). Accordingly, the sample was titrated with 0.025N sodium thiosulfate solution. During titration with sodium thiosulfate the golden color formed after the addition of sulfuric acid turned to pale yellow; therefore, for the precise determination of the end point, starch solution (0.02 g/ml) was added as an indicator. When the color of titrated solution became completely clear, sodium thiosulfate used in titration process was read from the burette. According to the reactions, each ml of Sodium thiosulfate is equal to 2/3 mg/L dissolved oxygen for 300 ml of water.

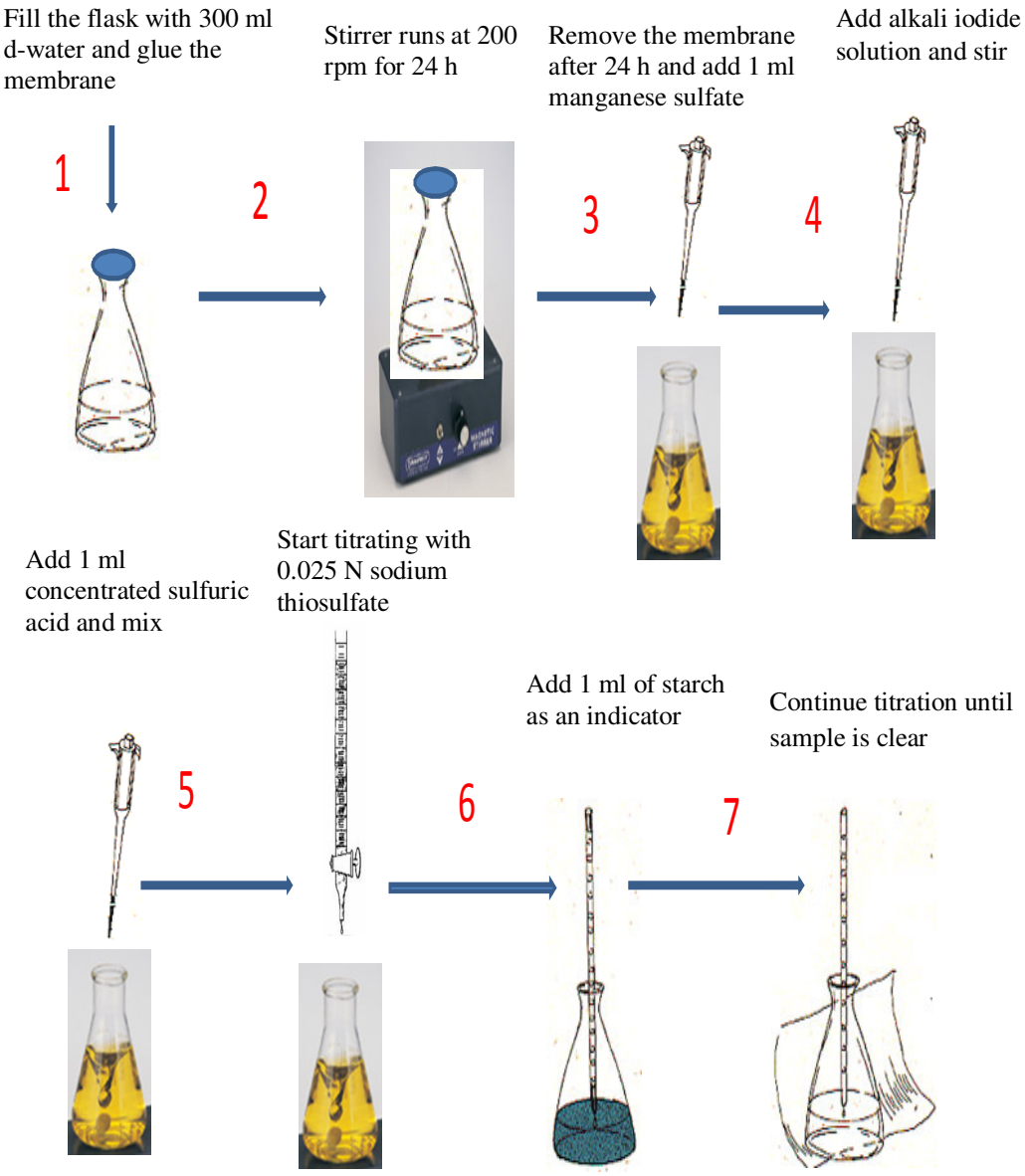


Figure 2.4. A schematic representation of the oxygen permeability test

(1) $Mn^{+2} + 2OH^- \rightarrow Mn(OH)_2$

(2) $2Mn(OH)_2 + \underline{1/2O_2} + H_2O \rightarrow 2Mn(OH)_3$

$(0.025/4)x\ 10^{-3}$ mol

(3) $2Mn(OH)_3 + 2I^- + 6H^+ \rightarrow 2Mn^{+2} + I_2 + 6H_2O$

(4) $I_2 + I^- \rightarrow I_3^-$

(5) $I_3^- + \underline{2S_2O_3^{-2}} \rightarrow 3I^- + S_4O_6^{-2}$

$0.025x\ 10^{-3}$ mol

Each ml of sodium thiosulfate (0.025 N) → m (O_2) = (0.025/4) x 10^{-3} mol x32 g/mol (O_2) = 0.2 x10^{-3} g.

For 200 ml of water→ $\frac{0.2\times10^{-3}\,g}{200\times10^{-3}\,L} = 1mg/L$ dissolved oxygen (DO) in water.

For 300 ml of water→ $\frac{2}{3}\times 1mg/L$ of DO exists in water.

Figure 2.5. Reactions involved in the determination of DO in water with respect to Winkler's method (http://www.lumcon.edu/education/studentdatabase/dissolved oxygen.asp)

2.2.2.5. Microbial Penetration

The ability of membranes to prevent microbial penetration was tested by attaching the membranes to the top of glass test tubes containing 20 ml of standard nutrient broth used as growth media in microbiological studies (Wittaya-areekul and Prahsarn, 2006). Before the tests, nutrient broth and glass test tubes were sterilized by autoclaving at 121 ^{o} C for 20 minutes. The negative control was a sterile nutrient broth in glass test tube closed with cotton ball and aluminum foil while the positive control was a sterile nutrient broth in test tube open to air. The test tubes were placed in an open environment for 1 week. The cloudiness of the nutrient broth in any tube was considered as microbial contamination. Also, spectrophotometric evaluation at 600 nm wavelength was carried out with µQuant^{TM} Microplate Spectrophotometer (Biotek Instruments Inc.. USA).

2.2.2.6. Mechanical Testing

The mechanical properties of membranes were evaluated by applying stretch test (tensile stress) with Lloyd LS500 Material Testing Machine (Lloyd, England) equipped with Nexygen computer software. Dumbbell sample pattern was used to prevent the slippage of membranes at the grip side during tests. For the same purpose, the ends of grips were covered with sandpaper. The gauge length (the length between the two grips) was 10 mm and the width was 4 mm. The stretch tests were carried out at test conditions of 25 $^{\circ}$C and 50± 3 % relative humidity. The crosshead speed of the system was adjusted to 10 mm/min to get a constant strain rate of 100 %/min. The results of tests were obtained as load versus deflection curves, which were then converted into stress-strain data by the computer program. Modulus of elasticity, ultimate tensile stress (tensile strength) and percent elongation at break were calculated from the stress-strain curves. At least six samples ($n \geq 6$) were used for each membrane type.

2.2.3. Biocompatibility Studies

Biocompatibility of membranes was studied with *in vitro* cytotoxicity tests using 3T3 fibroblast (An1 Swiss albino mouse fibroblast, obtained from Foot-and-Mouth Disease Institute of Ministry of Agriculture and Rural Affairs of Turkey) and keratinocyte (HaCat human keratinocyte, obtained from DSMZ, Braunschweig) cell lines. Light microscopy and SEM pictures were taken to examine the cell-biomaterial interactions (i.e. cell morphology; adherence onto membranes, distribution-spread, roundness etc.). MTT tests were also conducted to study the cell proliferation on membranes

2.2.3.1. Cell Culture Studies

2.2.3.1.1. 3T3 Fibroblast Cell Line Culture

The 3T3 fibroblast cell line was cultured in Dulbecco's modified Eagle's medium (DMEM high glucose-glutamine) supplemented with fetal bovine serum (FBS, 10 %, v/v) and penicillin/streptomycin (10 U/ml) at 37°C under humidified atmosphere of 5 % CO_2- 95 % air in incubator (5215, SHEL LAB, USA). The medium was refreshed 2 times a week. When the cells reached at least 80-90 % confluency, they were trypsinized with trypsin-EDTA (0.1 % in PBS) and passaged in 1:3 ratios.

2.2.3.1.2. Keratinocyte Cell Line Culture

The HaCat keratinocyte cell line was cultured in DMEM/Ham's F-12 supplemented with fetal bovine serum (FBS, 10 %, v/v) and penicillin/streptomycin (10 U/ml) at 37°C under humidified atmosphere of 5 % CO_2- 95 % air. The medium was refreshed 2 times a week. When the cells reached at least 80-90 % confluency (approximately 7 days), they were first rinsed with EDTA (0.05 % in PBS) and incubated in EDTA (0.05 %) for 10-20 minutes at 37°C and then trypsinized with trypsin-EDTA (0.1 % in PBS) for 5-10 minutes and passaged in 1:5-1:10 ratios.

2.2.3.1.3. Cell Seeding onto Membranes

Different membrane groups were held in glycine solution (0.2 M) in d-water for 30 minutes to block the unreacted GTA residues (Chena et al., 2007; Rho et al., 2006; Adekogbe and Ghanem, 2005). After drying the membranes, they were put onto previously sterilized cover slips (sterilized in 200 ^{o} C oven for 2 hours) that were placed in 24 well plates. Finally, the whole system was sterilized by overnight incubation in 70 % ethyl alcohol containing penicillin/streptomycin (0.1 %). The residual alcohol was rinsed two times with sterile d-water and they were left to dry.

Just before seeding, the samples were also exposed to UV light for 30 minutes. The membranes were seeded at a density of $3x10^4$ cells/well.

2.2.3.1.4. Microscopic Examination

The membranes seeded with cells were analyzed with light microscopy and photographed after both incubation times; 1 and 7 days. 3T3 cells and keratinocytes were evaluated in terms of morphological changes and degree of attachment to the membrane surface by microscopic examination (Nikon Eclipse TS100, China).

2.2.3.1.5. Proliferation Studies

The biocompatibility of the membranes was also evaluated in terms of proliferation of the cells on the membranes using MTT cell viability assay. This method is based on reduction of a tetrazolium salt by mitochondrial dehydrogenases to a dark blue formazan product (Mossmann, 1983). The extent to which MTT was reduced to a formazan product has been correlated with the cell viability.

At the end of each time period (1, 4 and 7 days), the density of viable cells were determined with MTT assay. Accordingly, the medium was removed and 500 µl MTT solutions (5 mg/ml in DMEM low glucose) was added to each well and incubated for 3 hours at 37 ^{o} C in a dark environment. After removing the MTT solution and washing with PBS, membranes were transmitted to other plates containing 500 µl dimethyl sulfoxide (DMSO) to solubilize the formazan crystals formed inside the cells. The absorbance was measured at 550 nm wavelength using µQuant^{TM} Microplate Spectrophotometer (Biotek Instruments Inc.. USA).

2.2.3.1.6. Scanning Electron Microscopy (SEM) Analysis

The membranes were initially coated with gold and the surface properties were analyzed with Scanning Electron Microscope (JSM-6400 Electron Microscope, JEOL Ltd., Japan) at the Department of Metallurgical and Materials Engineering in METU. Additionally, for biocompatibility studies the cells on the membrane surface were fixed with GTA solution (3 %) for 1 hour and gold coated before SEM examination.

2.2.4. Statistical Analysis

In comparing the groups for a single parameter One-way Analysis of Variance (ANOVA) test was done with Tukey's Multiple Comparison Test for the post-hoc pairwise comparisons using SPSS-9 Software Programme (SPSS Inc., USA). Differences were considered significant for $p < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Evaluation of the Experimental Conditions for Preparation of Sericin-Collagen Membranes

It was found that sericin and collagen had the highest solubility in acetic acid among different solvents used (i.e. water, acetic acid and organic solvents) under the requirement that the same solvent is to be used for both sericin and collagen in membrane preparation.

During optimization experiments, it was also recognized that further homogenization was required to increase the solubility of both proteins. Other treatments for this purpose involved centrifugation to remove undissolved particulates, and filtration to eliminate all intruders such as dust and dirt from the solution. Sericin solution was similarly processed except for centrifugation and homogenization with metal blender steps.

Non-sticky surfaces like Teflon were tried for casting membranes. For instance, Teflon tray, Teflon mold and Teflon sheets were investigated for their applicability. Among these surfaces Teflon tray was found quite satisfactory in that it was smooth enough to distribute the solution homogenously. Upon selecting the Teflon tray, cast solution was air dried and an intact membrane was easily peeled off the surface. Finally, to increase the rate of drying, the cast solution was put in an oven set at 37°C. However, this step was also eliminated due to the formation of air bubbles.

As a consequence, the experiments conducted in the laboratories of Imperial College and METU Engineering Sciences Department indicated the fact that collagen-sericin

membranes could easily be obtained from non-sticky surfaces like Teflon and the influence of the evaporating conditions on membrane structural properties should also be considered.

3.2. Selection of Materials and Adjustment of Membrane Thickness for Wound Dressing Applications

Sericin has many important properties which could make it a suitable candidate for biomedical applications. These are excellent oxygen permeability, antioxidant action, moisture regulating and antibacterial ability, UV resistance, anticancer and anticoagulant property (Kato et al., 1998; Murase, 1994; Nagura et al., 2001; Kurioka et al.,2004; Zhang et al., 2002; Cho et al., 2003; Tamada, 1997; Zhaorigetu, 2003). Various possible application areas of sericin such as cosmetics and biomedical applications (i.e. as degradable biomaterials, compound polymers, functional biomembranes, hydrogels, functional fibers and fabrics, etc) were reviewed by Zhang (2002) and Kundu et al. (2008) in detail. Nonetheless, it is estimated that 1 million tons of cocoons (fresh weight) is produced worldwide and 50,000 tons of sericin from 400,000 tons of dry cocoon is depleted as a waste product (Zhang, 2002). Therefore, exploiting sericin in biomaterial applications such as wound dressing would provide a significant economic and medical benefit. The studies carried out to determine the effect of sericin on wound healing and its potential as a wound dressing gave promising results. The effect of sericin on the attachment of primary cultured human fibroblasts was studied by Tsubouchi et al. (2005) and it was found that the attachment of these cells was enhanced with sericin. The studies conducted by Aramwit and Sangcakul (2007) about the wound healing effect of sericin cream in rats suggested that sericin has wound healing effects without causing any allergic reactions. Tsubouchi (1999) developed a fibroin-mixed-sericin wound dressing that could accelerate healing and be peeled off without disturbing the newly formed skin. One of the disadvantages of sericin reported was its being the causative agent for immune responses to silk, but only when it was used in conjunction with fibroin. Yet, sericin by itself did not elicit an immune response (Panilaitis et al., 2003).

In addition to being used for various drug delivery systems collagen was also used for tissue engineering applications including skin replacement, bone substitutes and artificial blood vessels and valves, tendon and ligament, peripheral nervous systems, cartilage and meniscal tissue. (Lee et al., 2001; Guelcher and Hollinger, 2006). Collagen is chemo-attractive to cellular elements of healing such as granulocytes, macrophages and fibroblasts. Collagen provides a template for cellular attachment, migration and proliferation (Sai and Babu, 2000). Cells such as fibroblasts and keratinocytes specifically recognize collagen substrates (Silver, 1994). Reconstituted type I collagen is known as a suitable material for skin replacement and burn wounds due to its mechanical strength and biocompatibility (Rao, 1995).

Collagenous matrices are usually stabilized by cross-linking to maintain their stability during implantation (Jorge-Herrero et al., 1999; van Wachem et al., 1999). Reconstituted collagenous matrices stabilized by cross-linking are used widely in the biomedical field (Yannas, 1992; Silver, 1994, Chvapil et al., 1991; Schroeder et al., 1997). Although collagen has been efficiently cross-linked with physical methods such as ultraviolet/gamma- ray irradiation and dehydrothermal treatment (Lee et al., 2001), Glutaraldehyde (GTA) is the most extensively used reagent for cross-linking collagen (van Luyn et al.,1995). Also, Zhang (2002) stated that sericin could be cross-linked or blended with other polymers to form an intact structure. However, GTA is known to elicit cytotoxicity, and calcification of GTA treated collagen on implantation have caused great concern (van Luyn et al.,1995). Charulata and his colleague Rajaram (2003) evaluated the cross-linking effect of GTA and found that GTA had superior cross-linking density results, but the time of cross-linking treatment greater than 2 hours did not enhance the cross-linking density. Therefore, in our study 2 hour cross-linking treatment was used. The cross-linker concentration of 3 % was assumed to be optimum for our study, because the test results of Wittaya-areekul and Prahsarn (2006) suggested that weakly cross-linked films with 3 % GTA did not contain any residual GTA and had sufficient integrity.

In order to understand whether increasing the membrane thickness would improve the membrane properties, thick membranes with the same protein ratios were also

prepared. Cut off point for optimizing membrane thickness was that it was not desired to produce hard and inflexible membranes. The thicknesses of the thick membranes were compared with those of thin ones in Table 3.1. For all the groups of thin membranes, the same total amount of protein (36 mg) was casted on the same surface area. Therefore, their thicknesses were very close (approx. 10 μm). However, for thick membranes the total quantity of protein casted on the same surface area changed with the weight proportion of sericin and collagen in membranes. For instance, 1/2-x and 2/1-x groups contained the same quantity of polymer (360 mg), but 1/1-x and C-x had 240 mg and 480 mg, respectively. The thickness variations between thick groups could be explained with the difference of their total polymer quantity.

Table 3.1. Thicknesses of membranes prepared with different ratios of sericin and collagen.

Sericin/Collagen (weight ratio)	**Average thickness of thin membranes** (μm)	**Average thickness of thick membranes** (μm)
C-x	9.67±2.31	34.25 ± 2.87
1/2-x	12.75 ± 0.96	19.25 ± 0.96
1/1-x	10.50 ± 0.71	17 ± 1
2/1-x	11 ± 1.41	23.75 ± 2.22

Membranes prepared from pure collagen solutions were indicated with "C". Membranes treated with cross-linker were shown with "x".

3.3. In Situ Characterization Results

3.3.1. Membrane Water Absorption

With water absorption experiments it was realized that cross-linked membranes had higher weekly water uptake values than uncross-linked ones (Figure 3.1). This could be explained with the fact that cross-linked membranes were less susceptible to hydrolytic degradation than uncross-linked ones. Therefore, it could be said that cross-linking treatment resulted in increased structural stability and water uptake. As opposing to this result, it was shown that GTA caused a reduction in swelling ratio of collagen matrices (Charulatha and Rajaram, 2003; Ma et al., 2003; Chen et al., 2007) such as porous sponges and scaffolds. The large pore spaces were filled with GTA and the free space was reduced as a consequence. Y.C. Wang et al. (2003) enumerated the parameters that affect the swelling ratio of a matrix as hydrophilicity, stiffness and pore structure. However, the membranes produced in this study did not have porous structure and therefore strongly supported that degree of swelling was more dependent on the increased hydrophilicity of the membranes as well as their structural stability rather than their porosity. Prolonging the incubation time in aqueous media showed that uncross-linked groups could not maintain their structural integrity after 2 weeks.

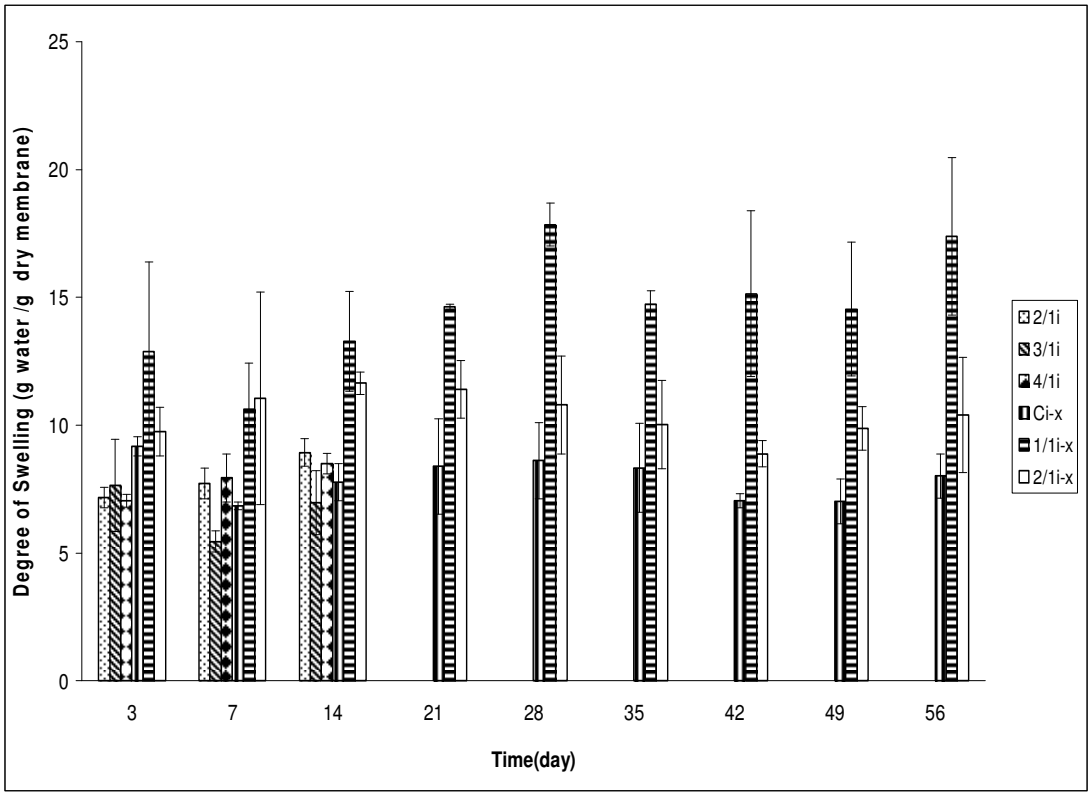


Figure 3.1. Degree of swelling of membranes for 8 weeks. Data are expressed as mean ± SD (n = 2).

The preliminary water absorption tests showed that membranes reached an equilibrium degree in swelling ratios at about 2 weeks. Similar EDS characteristics were observed for both thin and thick membranes after 2 week incubations. As seen in Figures 3.2 and 3.3, 1/1i-x and 1/1k-x had the highest EDS values. All sericin containing groups had higher EDS values than the ones containing only collagen (C-x). It is known that sericin is more hydrophilic than collagen. Kweon et al. (2001) stated that sericin had hydrophilic properties due to the presence of several hydroxyl groups. In addition, sericin consists of about 30 % serine, which is the main amino acid of natural moisture factor (NMF) in human skin (Nagura et al.,2001, Kurioka et al.,2004). This amino acid makes sericin an excellent moisturizing agent (Zhang et al.,2002; Cho et al., 2003). Yoshii et al. (2000) produced a blended hydrogel made of sericin, fibroin and PVA. This hydrogel was shown to have excellent moisture

absorbing and desorbing properties. Similarly, polyurethane foams containing sericin had excellent moisture absorbing and desorbing properties as well (Nomura et al., 1995). Therefore, the experimental results that thin and thick membrane groups containing sericin (1/1-x) had significantly higher EDS values than the groups comprising only collagen was compatible with one of the inherent properties of sericin. Also, among the cross-linked membrane groups a trend was observed as shown in the Figures 3.2 and 3.3. Accordingly, adding sericin into collagen membranes enhanced EDS clearly but increasing the ratio of sericin further (2/1, sericin/collagen) decreased EDS. The decrease was thought to be due to decrease in structural stability of the membranes.

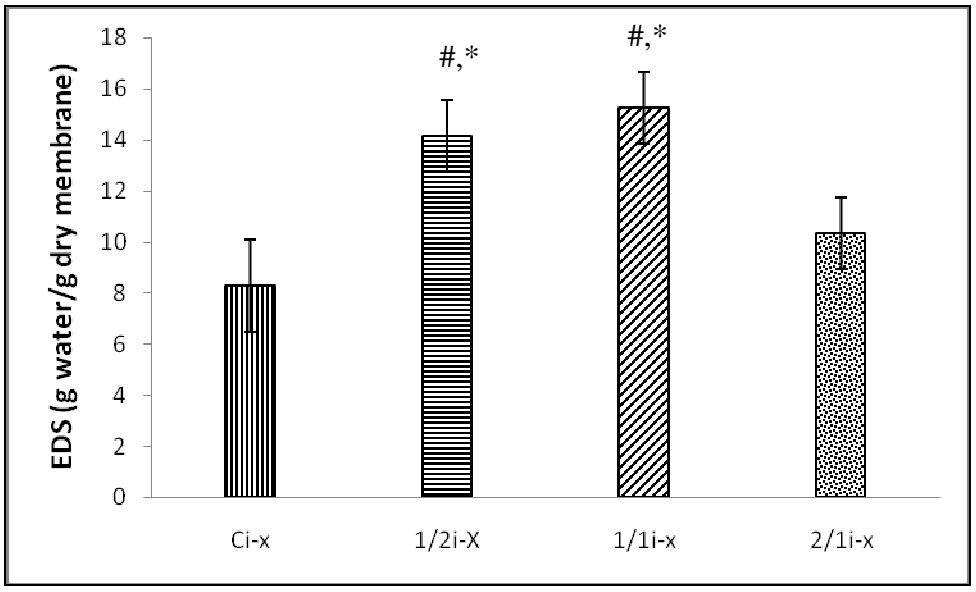


Figure 3.2. EDS measurements of thin membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD (n = 4). #: Significant difference from the group containing only collagen (Ci-x). *: Significant difference from 2/1i-x. $p < 0.05$.

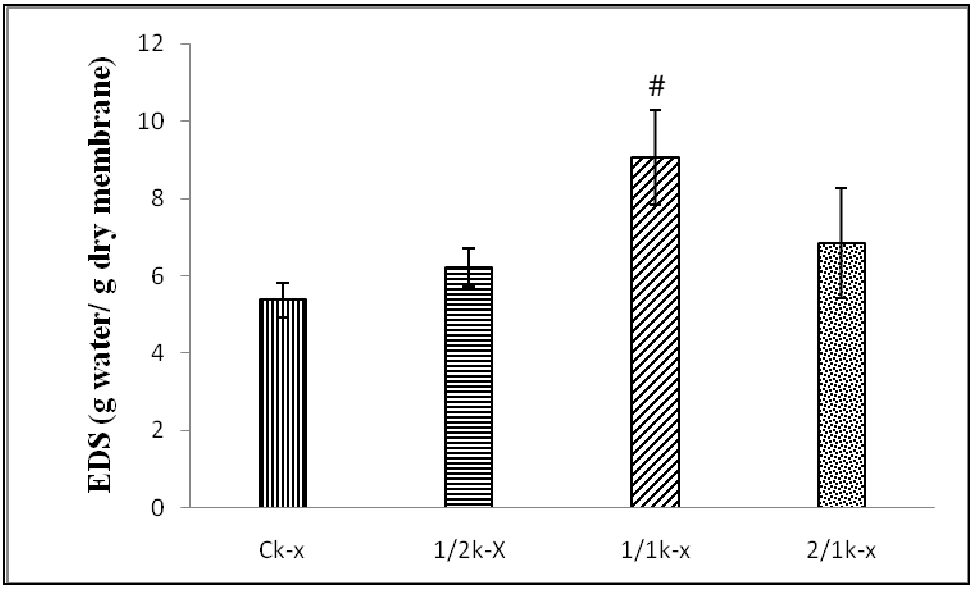


Figure 3.3. EDS measurement of thick membranes containing only collagen (Ck-x), and different weight ratios of sericin/collagen (1/2k-x, 1/1k-x, 2/1k-x). Data are expressed as mean ± SD (n = 4). [#]: Significant difference from the group containing only collagen (Ck-x) $p < 0.05$.

L. Ma et al. (2003) specified an optimum value for fluid absorption of the scaffold that will be used for skin tissue engineering. Accordingly, a scaffold that absorbs fluid to about 80 times its initial weight is sufficient. However, swelling results from other studies showed large variations, and none of them reached to the optimum value determined by L. Ma et al. GTA cross-linked collagen and chitosan films fabricated by casting method similar to the method used for sericin/collagen membranes had swelling ratios of 0.4-0.7 (g/g) (Shanmugasundaram et al., 2001). L. Ma et al. fabricated freeze-dried chitosan-collagen scaffolds and showed that scaffolds cross-linked with GTA had twice the swelling ratio of uncross-linked ones (160 and 80 g/g, respectively). PGA-chitosan hybrid matrices produced by Wang et al. (2003) had stable swelling ratios of 2.4-3.1(g/g). J. Ma et al. (2001) investigated the effect of porogens on the swelling ratio of chitosan bilayer scaffold and had a range of 1-2.6 (g/g). EDS of collagen/chitosan nanofibrous membranes prepared by Chen et al. (2007) were 9 (g/g) but decreased to 2.7(g/g) after cross-linking with GTA. EDS of different chitosan wound dressings produced by Mi et al. (2001) had a

range of 7.6-1.8. Wound dressing sponges containing different weight proportions of gelatin and alginate had water uptake values in the range of 26-35(g/g) (Choi et al., 1999). Kokabi et al. (2007) assumed that the EDS range of 4.5-3.5 (g/g) that was reached with PVA-clay wound dressings were enough to be used as a suitable wound dressing. Chitosan-polysaccarides composite wound dressing films produced by Wittaya –areekul and Prahsarn had EDS range of 4-7 (g/g) which was very similar to sericin-collagen membranes. Chitosan wound dressings cross-linked with GTA had EDS range of 29.9-31 (g/g) (Adekogbe and Ghanem, 2005). Hence, the EDS values of both thin and thick sericin-collagen membranes prepared in this study were comparable with those in literature and were thought to be in acceptable range for EDS for wound dressing applications.

3.3.2. In Situ Degradation

The preliminary membrane preparation studies conducted in Imperial Collage suggested that sericin/collagen membranes up to an increasing sericin ratio of 4:1 could be prepared. In order to increase the membrane resistance to degradation and to enhance its mechanical properties it was thought that cross-linking the membranes with GTA solution would be beneficial for wound dressing applications. GTA follows a reaction pathway through cross-linking its aldehyde groups with the amine groups of lysine or hydroxylysine residues (Sheu et al., 2001). Although GTA is associated with marked cytotoxicity (Huang-Lee et al., 1990; Petite et al., 1995) cross-linking treatment with GTA influences the physical properties of collagen matrices in desirable ways (Charulatha, Rajaram, 2003). The effect of GTA on stress-strain characteristics and resistance to collagenase digestion of collagen were evaluated by Charulata and his colleague. GTA treated collagen was found superior for its increased tensile strength and high resistance to collagenase degradation. It must be noted that the spatial orientation of amino acid side chain residues on collagen plays an important role in determining the cross-linking density and consequent physical properties of the collagen matrix.

It was seen that all cross-linked sericin-collagen membranes maintained their structural integrity during 7 weeks of *in situ* incubations, with the high degree of weight loss in groups containing sericin. The uncross-linked groups lost their structural integrity almost completely after 2 weeks; therefore, the weight loss of membranes was higher for uncross-linked groups (Table 3.2).

Table 3.2. Weight loss of sericin-collagen membranes after 7 week incubations in d-water.

Sericin/Collagen (w/w)	**Initial Average Weight (mg)**	**Average Weight Loss (%)**
2/1i*	5.0 ± 0.00	78 ± 0.00
3/1i*	5.3 ± 0.36	84.82 ± 1.67
4/1i*	4.7 ± 1.27	85.39 ± 2.06
Ci-x	2.3 ± 0.28	10.38 ± 7.95
1/1i-x	1.9 ± 0.14	33.61 ± 16.11
2/1i-x	2.3 ± 0.28	31.90 ± 11.45

*2/1, 3/1, 4/1 membranes lost their structural integrity to a great extent during 2 weeks of incubation (data on the table above), therefore the degradation measurements for those membranes were done before 7 weeks. The letter "i" represents thin membranes. Membranes prepared from pure collagen solutions were indicated with "C". Membranes treated with cross-linker were shown with "x".

As expected, the membrane resistance to hydrolytic degradation increased by cross-linking with GTA. However, the cross-linking effect of GTA on sericin and collagen was unknown. Sericin has hydrophilic properties due to the presence of several hydroxyl groups (Kweon et al., 2001), on the contrary the side groups of amino acids of collagen are highly non-polar in character and hence hydrophobic (Park and Bronzino, 2003). It could be assumed that increasing the sericin ratio would probably

increase the hydrophilicity. Accordingly, as seen in Table 3.2, as the sericin ratio increased in uncross-linked groups, weight loss also increased. However, sericin contains large amount of amino acids with neutral polar functional groups which might have enhanced its cross-linking efficiency with GTA. This might have reversed its effect in terms of degradation in cross-linked groups. Although the combined effect of cross-linking treatment and hydrophobicity of individual proteins was confusing, it seems that there is a trend for increase in degradation in terms of weight loss of membranes containing sericin. Using these initial optimization studies on degradation and stability four major groups were selected and prepared as thin and thick membranes for comparison of their *in situ* degradation properties for 4 weeks period. Accordingly, none of the cross-linked groups degraded significantly during 4 weeks period as determined from the previous degradation results (Table 3.3).

Table 3.3. In situ degradation results of sericin-collagen membranes for 4 weeks in d-water.

Sericin/collagen (weight ratio)	**Average weight loss of thin membranes (%)**	**Average weight loss of thick membranes (%)**
Cx	10.28 ± 6.61	14.89 ± 2.82
1/2x	23.37 ± 5.88	10.57 ± 1.63
1/1x	23.16 ± 3.73	17.66 ± 2.74
2/1x	18.04 ± 5.22	22.70 ± 1.93

Membranes prepared from pure collagen solutions were indicated with "C". Membranes treated with cross-linker were shown with "x".

Material degradation can result from biological processes such as enzymatic degradation or environmentally induced degradation from mechanical, metal-catalyzed oxidation, and from the permeation of body fluids into the polymeric

devices (Bruck, 1991). It is known that a number of cell types such as polymorphonuclear leukocytes, fibroblasts, and macrophages, during the wound healing period, are capable of secreting enzyme collagenases which cleave a collagen molecule at 1/4 position from the C-terminal end of the molecule (Woolley, 1984).

If only the hydrolytic degradation results are evaluated it looks that the cross-linked membranes produced in this study might be considered as suitable candidates for chronic wounds where an 8-week degradation time is recommended. However, it is known that biodegradation tests would obviously be different than these results, because wound dressings will not only be exposed to hydrolytic degradation, but also to enzymatic degradation on the wound area. Therefore, the stability of membranes will probably decrease when placed on a wound site. The biodegradation time for about 25 days is suggested for a good skin scaffold. A more rapid biodegradation rate would reduce the scaffold within a few days to the liquid state, rendering it ineffective as a wound closure device. On the other hand, a scaffold that degrades only minimally within 3–4 weeks would hinder the wound healing process (Yannas et al., 1980). In this part it was aimed to compare the effects of sericin and cross-linking agent addition on degradation behaviour of sericin-collagen membranes. However, these results are not absolute for the use of these membranes and for complete conclusion, *in vivo* degradation studies need to be done.

3.3.3. Water Vapor Transmission Rate (WVTR)

WVTR values for all groups of thick membranes (Figure 3.4) were found similar with being statistically indifferent. However, values had more variation among thin membranes with 1/2i-x group having the highest WVTR (1084.03 g/ m^2 /day). This group had statistically significant difference with only 1/1i-x group (1003.57 g/m^2/day). No direct relationship could be found between the ratio or protein types and WVTR. But the thickness change of membranes was effective on WVTR. When the thickness of a membrane increased, WVTR of it decreased significantly in all composite groups. This was not traced for sericin/collagen membranes, 1/1i-x and1/1k-x. Yet, the thickness difference was not very high for this group. As a

result, it was thought that WVTR could be controlled by adjusting the membrane thickness but not the ratio of the proteins.

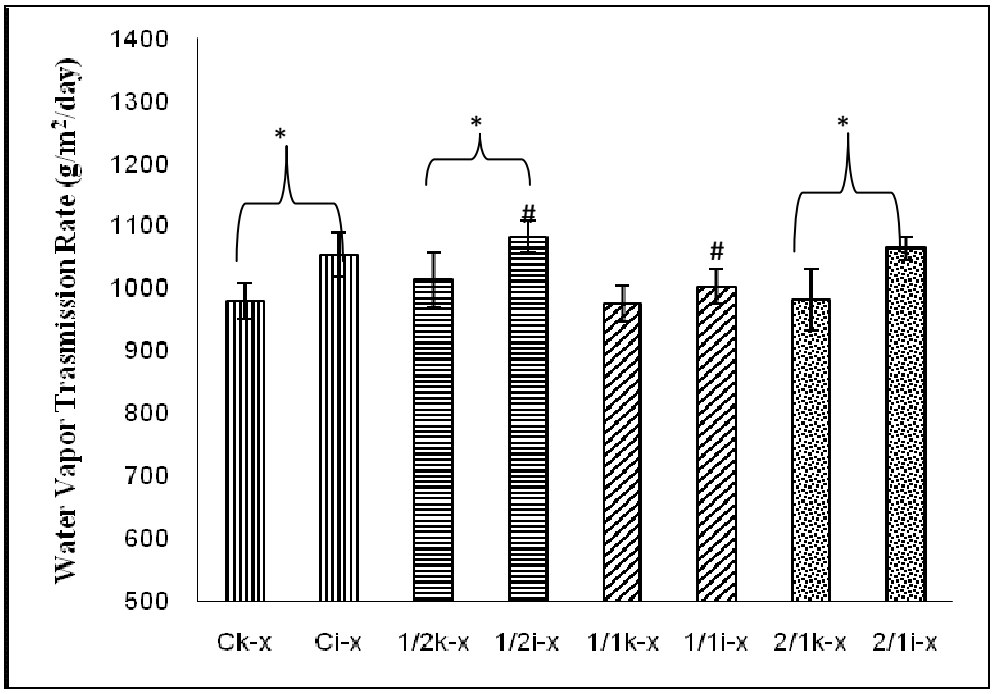


Figure 3.4. Comparison of water vapor transmission rates of cross-linked thick and thin collagen (Ck-x, Ci-x) and sericin/collagen (1/2k-x, 1/2i-x, 1/1k-x, 1/1i-x, 2/1k-x, 2/1i-x) membranes. Data are expressed as mean ± SD ($n \geq 4$). $^{\#}$: Significant difference between the groups of thin membranes. *: Pairwise comparisons between thin and thick membranes of the same groups. $p < 0.05$

Actually, a moist environment is desired for quick healing of wound, but at the same time excessive pooling of exudates below the wound bed should be avoided to decrease the risk of infection. On the other hand, wound dehydration would result in death cell debris resembling a scar tissue. Therefore, a dressing that could control the evaporative water loss from wound at an optimal rate is required. The rate for normal skin is 204 g/m^2 per day, while that for injured skin can range from 279 g/m^2 per day for a first-degree burn to 5138 g/ m^2 per day for a granulating wound. Owing to the fact the WVTR varies with respect to the type of wound, it might be suggested that

the range 980.90-1084.03 g/m^2/day measured for the sericin-collagen membranes is likely to be suitable for low to medium exudating wounds (Mi et al., 2001; Yannas et al., 1980).

3.3.4. Oxygen Permeability Test Results

Oxygen permeability tests were based on measuring the amounts of dissolved oxygen in the media isolated from air by the test membranes separately. Hence, the measured parameter, i.e. the amount of dissolved oxygen, was considered to be a direct result of oxygen permeability character of the membranes. Normally, the dissolved oxygen (DO) in water is influenced by temperature, atmospheric pressure and the amounts of dissolved solids (salinity). Increasing temperature or salinity reduces DO, whereas increasing pressure increases it. The oxygen permeability test was performed at room temperature (approx. 25 $^{\circ}$C) and under atmospheric pressure (less than the sea level<760 mmHg). Distilled water was used to eliminate the effect of dissolved solids (salinity was zero).

Oxygen permeability properties of membranes were compared by using the data in Table 3.4. The open control was measured to have a DO value of 8.31 ± 0.27 mg/L, but the closed control has as low DO as 6.95 ± 0.056 mg/L. The closed group had significantly lower DO values than all the groups except for 1/1i-x and Ck-x. Closer the DO values of membranes to that of positive control, the better the oxygen permeability. The results suggested that most of the membranes were highly permeable to oxygen. However, in terms of statistical significance, only thin membrane groups (2/i-x, 1/2i-x) had as high DO values as the open control. Other membranes had DO values in between two controls denoting different degrees of oxygen permeability. DO results also showed that increasing membrane thickness caused a statistical decrease in membrane permeability to oxygen. There was no statistical difference between the membranes containing sericin/collagen ratios of 2/i-x, 1/2i-x. None of the thick membrane groups (Ck-x, 2/1k-x, 1/1k-x, 1/2k-x) had significantly high DO values as the open control. This showed that they were not as highly permeable as thin membranes to oxygen. Statistical analysis also showed that

membranes (2/1k-x, 1/1k-x, 1/2k-x) have DO significantly higher than the closed control. Only pure collagen membranes (Ck-x) have very low permeability values. As a consequence, all the membranes except Ck-x were found highly permeable to oxygen in comparison to the closed one. For thick membranes, only membranes (2/1k-x, 1/2k-x) had significantly higher OD than pure collagen membranes (Ck-x). For thin membranes, the membrane containing the highest sericin ratio (2/1i-x) had significantly higher DO than all other groups. It might be suggested that addition of sericin to the membranes could enhance the oxygen permeability provided that further tests were also made. In parallel to our results, Murase (1994) discovered that films made of sericin and fibroin had excellent oxygen permeability. It can be concluded that addition of sericin enhanced the oxygen permeability for both thin and thick membranes.

Table 3.4. Oxygen permeability results of thin and thick membranes.

Sericin/collagen (weight ratio)	**DO for thick membranes (mg/L)**	**DO for thin membranes (mg/L)**
Cx	7.25 ± 0.064^{a}	7.50 ± 0.36^{ab}
1/2x[c]	7.80 ± 0.13^{abd}	8.067 ± 0.067^{by}
1/1x	7.58 ± 0.13^{ab}	7.39 ± 0.21^{axy}
2/1x[c]	7.85 ± 0.33^{abd}	8.24 ± 0.24^{bex}
Open control	8.31 ± 0.27	
Closed control	6.95 ± 0.056	

Data are expressed as mean ± SD ($n \geq 4$).
[a] Significant difference from the open control at $p < 0.05$.
[b] Significant difference from the open control at $p < 0.05$.
[c] Significant difference between the same group of thin and thick membranes at $p<0.05$.
[d] Significant difference from the group containing only collagen at $p<0.05$ (Thick membranes).
[e] Significant difference from the group containing only collagen at $p<0.05$ (Thin membranes).
[x,y] Significant difference between the dual groups of thin membranes at $p<0.05$.

3.3.5. Microbial Penetration

Experimental results showed that none of the membrane groups allowed the penetration of microorganisms through the membranes for a week period (Figure 3.5). Optical densities of the tubes that were covered with membranes were statistically indifferent than the closed control and all of the membrane groups had O.D. values statistically lower than the open one (directly exposed to air). As also observed with physical examination of the tubes (Figure 3.6), nutrient broth provided an appropriate environment for microbial growth within the tubes. The microbial growth resulted in blurry media. Since sericin has antibacterial property (Zhang et al., 2002) it might have inhibited the intrusion of bacteria through membranes. However, there was also no microbial growth in pure collagen membranes. Thus, the overall results of membranes suggested that these results could be related to their dense structures. Therefore, the membranes were thought to behave like air filters preventing the passage of microorganisms rather than acting as antimicrobial agent for one week study period. The antimicrobial effect of sericin might be more clearly seen in a longer term observation or with *in vivo* studies. Therefore, this property of sericin was thought to bring extra advantage for membranes used in wound dressing applications compared to that of pure collagen membranes. According to previous works, it was also observed that even 64 layers of gauze cannot prevent the entry of exogenous bacteria into the wound (Sai and Babu, 2000). This fact showed one of the superiorities of membranes produced in this study.

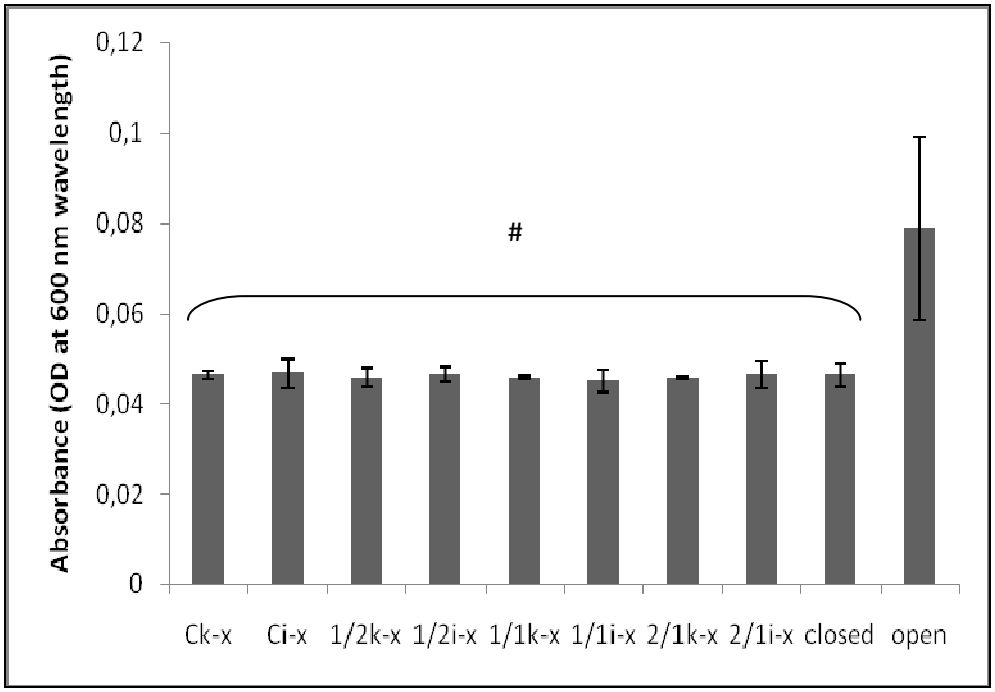


Figure 3.5. Absorbance (O.D. at 600 nm) measurement results of nutrient broths covered with thick and thin collagen (Ck-x, Ci-x) and sericin/collagen (1/2k-x, 1/2i-x, 1/1k-x, 1/1i-x, 2/1k-x, 2/1i-x) membranes after one week incubation time at RT. Controls are closed and open groups. Data are expressed as mean ± SD (n =3). $^{\#}$: Significant difference from the open control. $p < 0.05$.

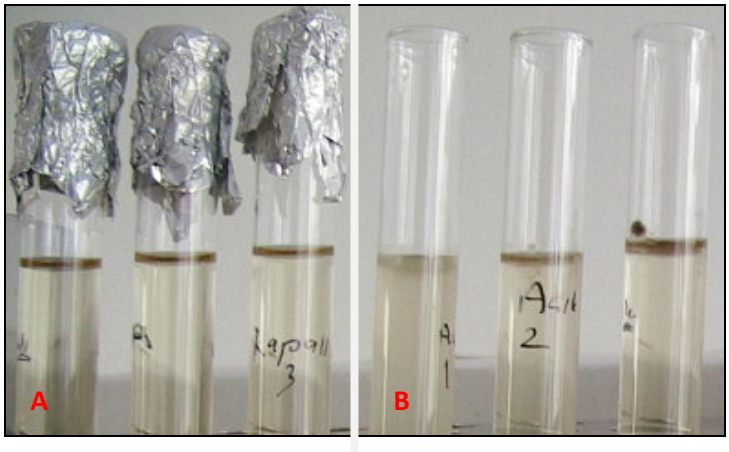


Figure 3.6. Bacterial penetration test tubes used as (A) closed and (B) open controls.

3.3.6. Mechanical Testing

A typical stress-strain curve for collagen fibers and soft collagenous tissues is such that initially the stress does not increase a lot while the strain does creating a toe region. This was previously explained in detail in the introduction part. Briefly, this behaviour is due to the relaxed fibers of collagen that have been cross-linked with either inter or intra-molecular linkages. In order to circumvent this toe region, the membranes were griped tautly between the jaws by exerting a pretension. This enabled the removal of undulation and initial alignment of the three-dimensionally organized collagen fibers along the tensile load direction, thereby eliminating the toe region (Figure 3.7). The general stress-strain behavior of membranes was explained in this figure. Accordingly, the crimping collagen fibers expand to such a great extent that the cross-links holding them becomes stressed, and the stress-strain curve becomes linear. This linear region is very similar to the stress-strain curve of elastic materials and the plot follows Hookean behavior. After a specific degree of tensile stress (proportional limit), the linear relationship between stress and strain is lost. Then the membrane behaves nonlinearly until reaching a maximum stress value (ultimate tensile stress or tensile strength) at or after which it breaks. It must be noted that, especially for the brittle membranes, pretension caused premature failures. In this study, pretension was lessened for such membranes, thereby allowing the formation of a toe region (Figure 3.8).

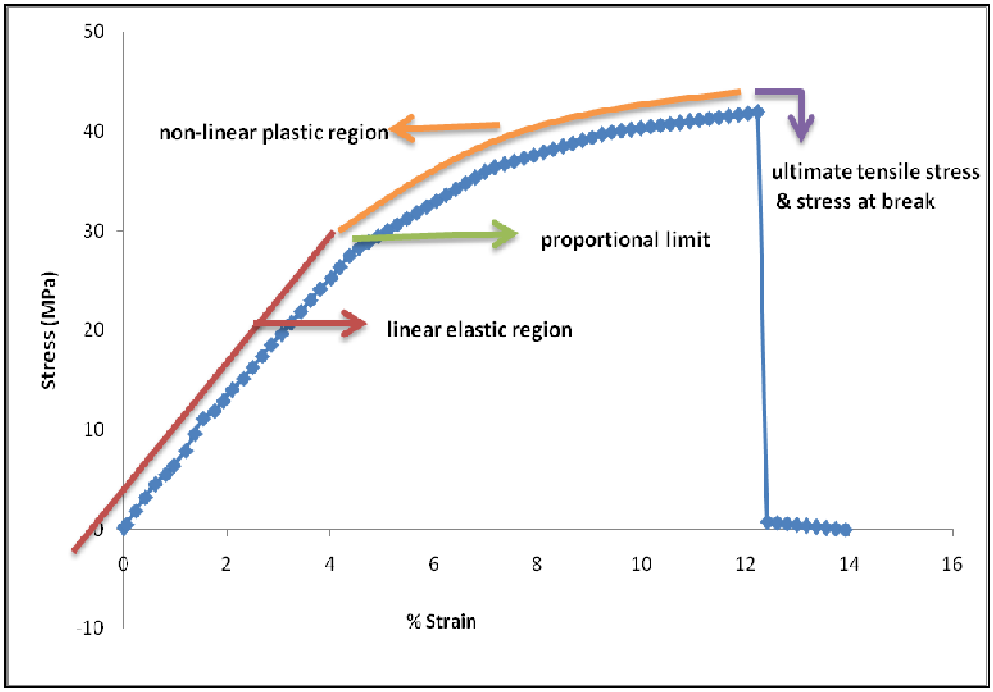


Figure 3.7. Representative stress-strain curve for collagen membrane (Ck-x) undergoing brittle failure

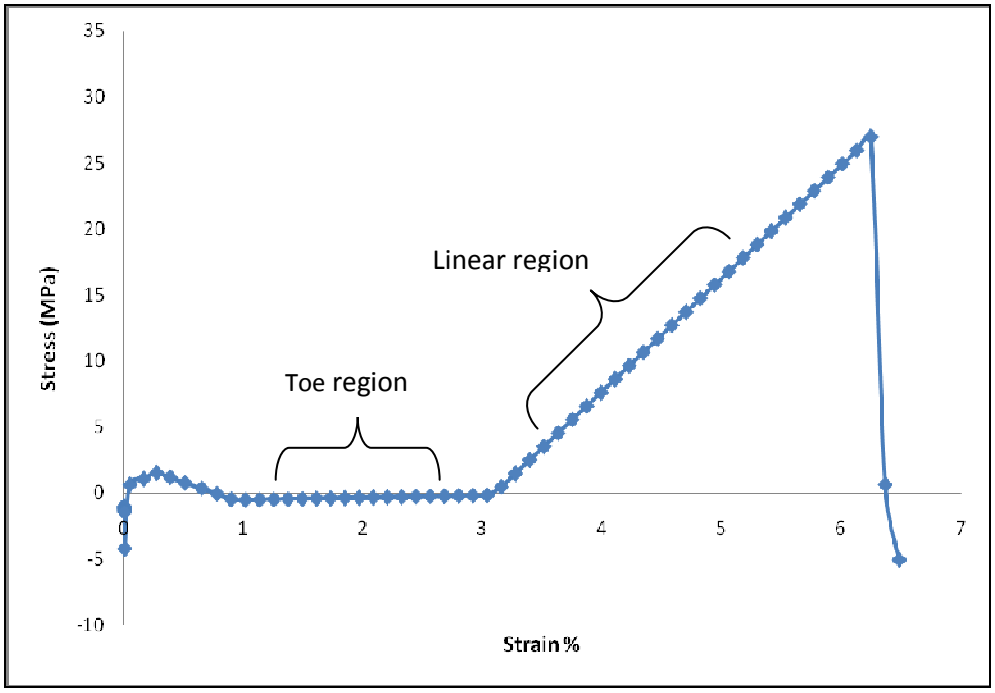


Figure 3.8. Stress-strain curve indicating the toe region in membranes obtained for 1/2k-x

In the stress-strain curves of sericin/collagen membranes generally the plastic region was vaguely realized or completely lost. The deformities occurred on the membrane surface during the membrane preparation sometimes caused abrupt decrease in stress during tensile stretching (Figure 3.9). To prevent this event, the membrane tensile testing environment was adjusted to have a relative humidity (RH) of 50 % ± 3 and 25 ^{o} C. Therefore, this adverse effect of deformities on membrane tensile testing was observed to decrease. The tensile test was accepted successful when the failure occurred in the middle of the gauge length of samples (Figure 3.10).

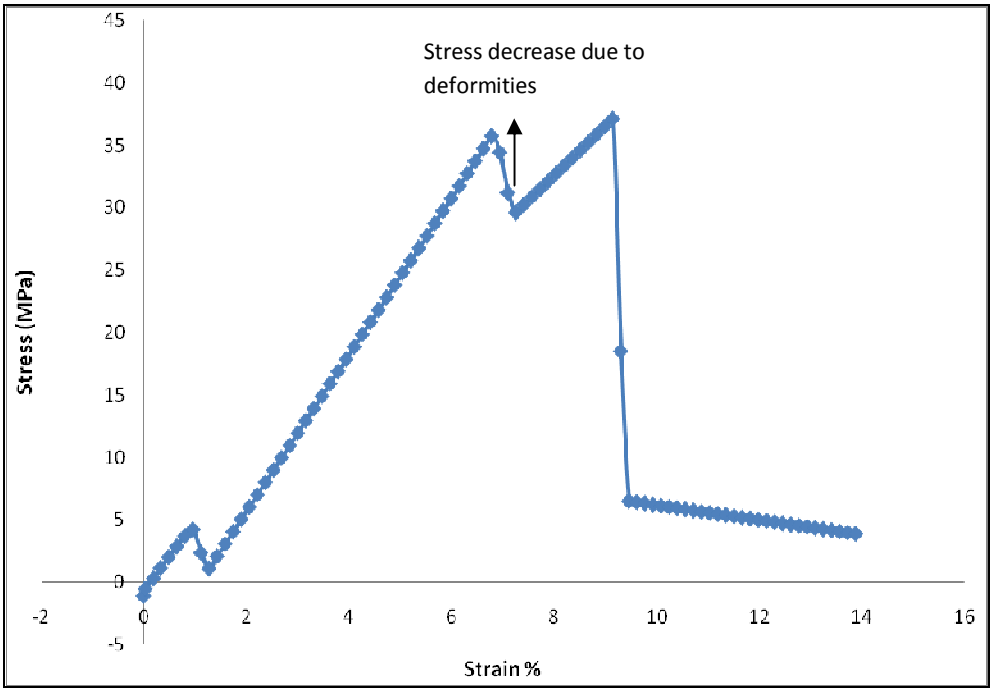


Figure 3.9. Stress-strain curve indicating the abrupt decrease in stress obtained for 1/2k-x

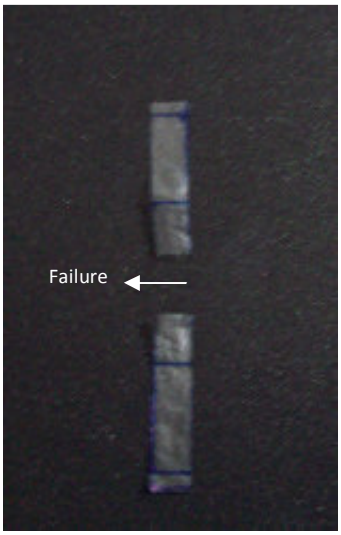


Figure 3.10. Successful tensile break occurred in the middle of the gauge length

Tensile strength is an intrinsic property of materials, and consequently does not depend on the size of the specimen. However, it is affected by the preparation procedures of the specimen and the temperature of the test environment and material. Hence, mechanical tests were carried out at humidified warm laboratory environment for all samples (http://en.wikipedia.org/wiki/Tensile_strength).

The ultimate tensile stresses (UTS) of thick membranes were measured to be between 24.93 ± 3.09 and 44.92 ± 3.72 MPa (Figure 3.11). Fung (1993) reported a tensile strength range of 50 to 100 MPa for collagen fibers. Meyers et al. (2008) found that the tensile strength of collagen was approximately 70–150 MPa. Also, the tensile strength of skin was shown as 7.6 MPa (Park and Lakes, 1992) and 2.5-16 (Silver, 1987) by researchers. Thick membranes produced using sericin and collagen had smaller ultimate tensile stress values than the reported values of collagen. With thin membranes of similar compositions lower tensile strengths (9.68 ± 3. 78 to 38.40 ± 5.45 MPa) were obtained (Figure 3.12). The difference of tensile strengths between the thin and thick membranes could be explained with the fact that the latter was denser. Pure collagen membrane (Ck-x) had significantly higher UTS than the other membrane groups (1/1k-x, 2/1k-x) except for the group containing the highest collagen ratio (1/2k-x). UTS of 1/2k-x group was also significantly higher than 2/1k-

x group. UTS of 1/1k-x membranes was neither statistically different than 1/2k-x nor 2/1k-x. Parallel results were obtained with thin membranes in terms of compositional effects; pure collagen membrane (Ci-x) had UTS significantly higher than all other membrane groups (1/2i-x, 1/1i-x, 2/1i-x). 1/2i-x group was statistically indifferent than 1/1i-x but higher than 2/1i-x. As a whole, there was a clear trend that UTS of membranes decreased when sericin was present in the membrane structure. The statistical difference was seen especially between the groups having the highest ratio difference of components. Due to the fact that collagen was derived from a reconstituted source and exposed to low pH solvent during membrane preparation steps, its triple helix structure was thought to be denatured. In addition to that, presence of sericin molecules between the collagen fibers might have acted as defect sites making the membranes more brittle. For instance; glass fibers have a tensile strength greater than steel, but bulk glass usually does not. This is due to the stress intensity factor associated with defects in the material; the size of defect sites grows as the size of the sample gets larger (http://en.wikipedia.org/wiki/Tensile_strength).

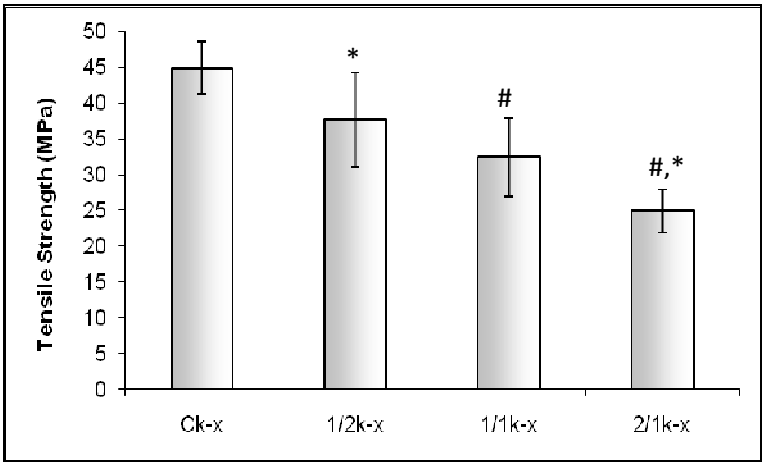


Figure 3.11. Comparison of tensile strength values of thick membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2k-x, 1/1k-x, 2/1k-x). Data are expressed as mean ± SD ($n \geq 6$). $^{\#}$: Significant difference from the group containing only collagen (Ck-x). *: Significant difference between the dual protein groups. $p < 0.05$.

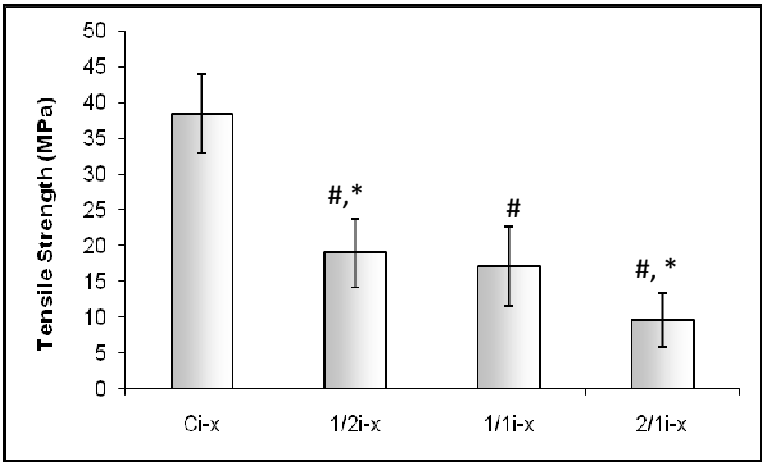


Figure 3.12. Comparison of tensile strength values of thin membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 6$). $^{\#}$: Significant difference from the group containing only collagen (Ci-x). *: Significant difference between the dual protein groups. $p < 0.05$.

Determination of the elastic modulus (E) is very important as it is the inherent property of material (http://en.wikipedia.org/wiki/Stiffness). Since extensibility is a selection criterion for wound dressings, a low modulus of elasticity (with high extensibility) was sought. In this study E was measured to range from 0.499 to 0.9 GPa for pure collagen membranes, thick and thin types respectively (Figures 3.13 and 3.14). This range was compatible with the E range of collagen fibers reported as 0.350 and 1 GPa (Fung, 1993; Gentlemen et al., 2003). In addition, the elastic modulus of collagen was determined as approximately 1–1.5 GPa (Meyers et al., 2008). Cross-linked dry collagen films of X.H. Wang et al. (2003) had E value of 1.16 GPa approximately, but decreased to 5.8 MPa in wet form. Besides that, E for skin found between 6-40 MPa (Silver, 1987), which was much lower than the individual collagen fibers. It is known that E changes with respect to direction of load applied when the material is anisotropic. The membrane samples were cut arbitrarily before tensile tests and the ones in the same batch of test did not have a significant E difference. Therefore, it can be suggested that sericin/collagen membranes were homogenous in the x-y plane which was parallel to the test

direction. The E of Ck-x membranes was significantly lower than those of 1/1k-x and 2/1k-x but statistically not different than the E values of 1/2k-x membranes. The E values of thick membrane groups, 1/2k-x, 1/1k-x and 2/1k-x did not have a significant difference but had an increasing trend with increase in sericin content (Figure 3.13). The E values of thin membrane groups were somewhat parallel with the thick ones except for Ci-x being significantly higher than only lowest sericin containing group (1/2i-x) (Figure 3.14). 1/2i-x membrane was also significantly lower than 2/1i-x. However, 1/1i-x group did not show a significant difference from any of the test groups.

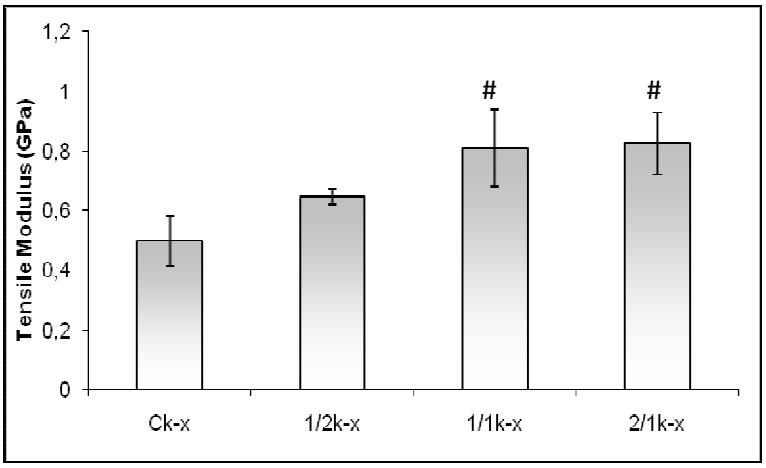


Figure 3.13. Comparison of tensile modulus values of thick membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 6$). $^{\#}$: Significant difference from the group containing only collagen (Ck-x). $p < 0.05$.

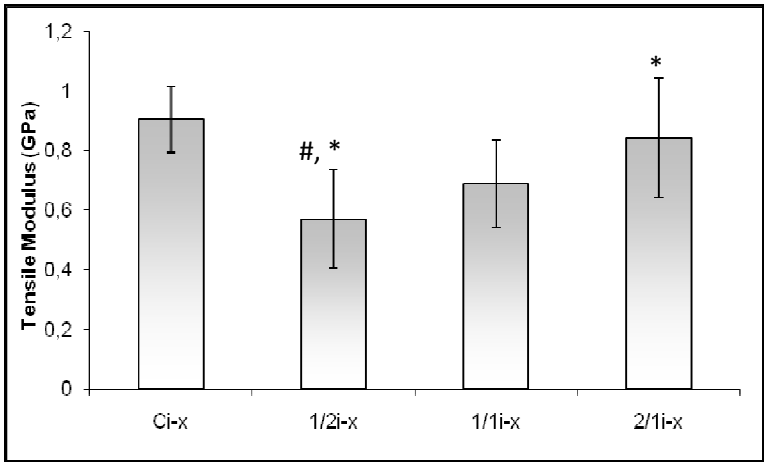


Figure 3.14. Comparison of tensile modulus values of thin membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 6$). $^{\#}$: Significant difference from the group containing only collagen (Ck-x). * Significant difference between the dual protein groups: $p < 0.05$.

What gives skin the high extensibility is elastin fibers. Ultimate percent elongations of collagen and elastin fibers were specified as 10 % and 100 %, respectively by Park and Lakes (1992). Although elastin fibers can elongate 100 %, ultimate elongation of skin is 78 % (Park and Lakes, 1992). The maximum strain of collagen was reported as in the 10-20 % range (Meyers et al., 2008). In this study, it was aimed to mimic the mechanical properties of skin with the membranes as much as possible. However, it was not possible to reach the reported elongation at break (EB) values with only collagen. In the present study it was found that the pure collagen membranes (Ck-x) could elongate as much as 12. 64 ± 1.26 % in dry form (Figure 3.15). This value was within maximum strain range of collagen (10-20 %) found by Meyers et al. (2008). On the contrary, the lowest EB (2.09 ± 0.53 %) was found for the membranes having the highest sericin ratio. Statistically, EB value of Ck-x membrane group was greater than all other groups of thick membranes. 1/2k-x membrane was greater than 1/1k-x and 2/1k-x groups for the same property. There was no statistical difference between the EB values of 1/1k-x and 2/1k-x groups. Similarly, EB of Ci-x was greater than all other groups of thin membranes (Figure 3.16). EB of 1/2i-x was significantly greater

than only 2/1i-x group. 1/1i-x did not significantly differ from 1/2i-x or 2/1i-x membranes. The obvious result of the statistical analysis was that as the sericin ratio was increased the EB of membrane tended to decrease. The EB range (2.09-12. 64 %) of membranes was soundly lower than that of skin. However, the EB of membranes are expected to increase in wet form. Also, the decreasing effect of cross-linking with GTA was shown by the studies of Charulatha, and Rajaram (2003). Accordingly, they showed that even though the tensile strength of collagen cross-linked with GTA increased, percent elongation had an opposite trend.

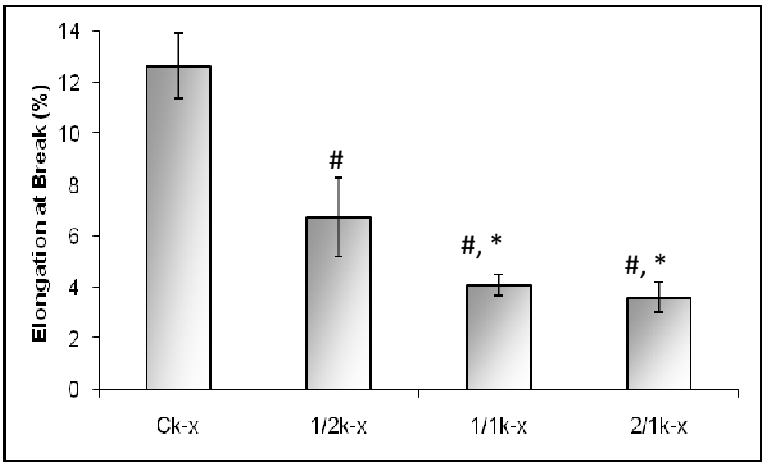


Figure 3.15. Comparison of elongation at break values of thick membranes containing only collagen (Ck-x), and different weight ratios of sericin/collagen (1/2k-x, 1/1k-x, 2/1k-x). Data are expressed as mean ± SD ($n \geq 6$). $^{\#}$: Significant difference from the group containing only collagen (Ck-x). *: Significant difference from 1/2k-x. $p < 0.05$.

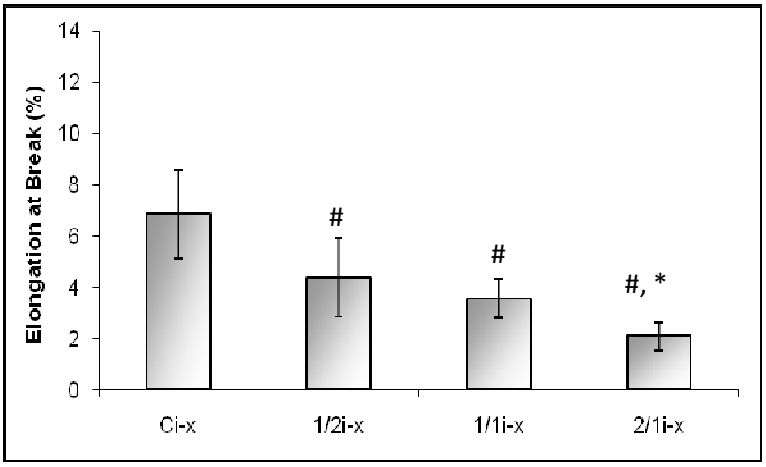


Figure 3.16. Comparison of elongation at break values of thin membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 6$). $^{\#}$: Significant difference from the group containing only collagen (Ck-x). *: Significant difference from 1/2i-x. $p < 0.05$.

PGA-chitosan blends prepared by Wang et al. (2003) had a maximum tensile stress of 3.30 ± 0.36 MPa and elastic modulus of 0.15 ± 0.05 GPa. Cross-linked collagen films of X.H. Wang et al. (2003) had tensile strengths of 33.5 ± 1.49 MPa and 2.25 ± 0.66 MPa, in dry and wet form respectively. Antibiotic-loaded collagen–hyaluronic acid matrices made by Park et al. (2004) had ultimate tensile strength range of 10-30 MPa. The nanofibrous collagen matrix that was produced by Rho et al. (2006) had tensile strength of 11.44 ± 1.20 MPa. This tensile strength value was compared with those of two commercial tissue regenerative membranes and thought to provide a similar level of mechanical stability. Chen et al (2007) produced collagen/chitosan nanofibrous membrane which had E (GPa) of 0.29 ± 0.04, and UTS (MPa) and tensile strain (%) of 1.35 ± 0.12 and 23.26 ± 1.22, respectively. In comparison to these studies, it could be suggested that sericin-collagen membranes had high tensile strengths, but low elongation. The low elongation could be explained with the GTA cross-linking (Charulatha, and Rajaram, 2003) and sericin addition. The mechanical properties of cross-linked collagen and B. Mori silk (with or without sericin) were given (Vepari and Kaplan, 2007; Altman et al., 2003; Meyers et al., 2008).

Accordingly, E, UTS and EB of cross-linked collagen were between 0.4-0.8 GPa, 47-72 MPa and 12-16 %, respectively. As seen, the results of this study in terms of cross-linked membrane produced are in agreement with the literature. These researchers also gave the mechanical properties of B. Mori silk (with and without sericin). UTS and E of silk with sericin were lower than of silk only; on the contrary, EB with sericin was higher. When sericin was added into collagen membranes UTS decreased similar to sericin containing silk fibroin. EB of collagen membranes also decreased as sericin was added into the collagen matrix while sericin had an opposite effect with silk fibroin. E of the collagen membranes containing sericin increased in contradiction to silk. Ki et al. (2007) discussed that the increase in EB of silk filament with increasing sericin ratio might be attributed to the lubrication and β-sheet forming effect of sericin. The increase in EB of silk (with sericin) could be related with the glue-like property of sericin. However, the glue-like property of sericin is attributed to the hydrogen bonding between serine residues of sericin with serine residues in the fibroin structural components of silk fiber (Huang et al., 2005). The gluing effect of sericin for sericin/collagen membranes was not likely due to lack of serine residues for collagen. Therefore, the EB of membranes did not increase when sericin is added; on the contrary, a reverse inclination was present. Consequently, the UTS results of our studies were parallel with the previous studies, but the different behavior for E and EB might have resulted from the different interaction of sericin and collagen as compared to silk fibroin and sericin. Actually, addition of sericin could be expected to bring about brittleness to membranes. Membranes with sericin were found more fragile in the dry state (Kundu et al., 2008). Yet, brittle structure of sericin could be alleviated by cross-linking (Nagura et al., 2001). In this study, the cross-linking treatment helped to preserve the membrane integrity. However, addition of sericin to collagen in the presence of cross-linker had no favorable consequences on extensibility of membranes.

In summary, addition of sericin to the membranes deteriorated the mechanical properties in terms of percent elongation and tensile strength. The membranes containing sericin also had high tensile strength in comparison to wound dressings produced in other studies. However, all sericin containing groups had significantly lower elongation percentages compared to pure collagen membrane. Considering that

the wound area is generally moist owing to the exudate discharge from the skin, it is thought that the dressing would get wet and become more extensible. Adekogbe and Ghanem (2005) showed that the elongation (%) of dry chitosan scaffolds increased from 20 to 80 when they got wet. In addition, EB of cross-linked dry collagen films of X.H. Wang et al. (2003) increased from 2.3 % to 36.5 % when they were wet. Hence, the measured strains of dry membranes are expected to increase in *in vivo* conditions. The molecular weight (MW) of type I collagen is about 283 kDa (Park and Bronzino, 2003). The molecular weight of silkworm sericin used in this study was given as 138 (kDa) (Capar et al., 2008). However, during initial treatments with acid and high temperature these values might have further decreased. It is known that the smaller the molecular weight of polymer the more difficult it will be to prepare the membranes. As sericin had smaller molecular weight compared to collagen, it was thought to decrease uniformity of membrane in terms of extended polymer chains. In addition, from microscopy analysis of membrane structure, round particles immersed in homogenous matrix in the case of membranes containing sericin was thought to be an indication of phase separation between two polymers. That phase-separated structure might also have decreased the mechanical properties of the membranes.

Another possible explanation for deterioration of elastic properties of membranes upon sericin addition might be the denaturation of secondary structure (beta-sheets) of sericin protein during either extraction from the silk or processing stages of membranes. Mechanical properties of sericin are related to β-sheet structures (Altman et al., 2003). Teramoto et al., (2005) showed that Sericin hydrogel is a β-sheet-rich structure containing strong intermolecular hydrogen bonds. It might be expected that the mechanical properties of sericin would weaken in case of sericin decomposition.

3.4. Biocompatibility of Sericin-Collagen Membranes

3.4.1. In Vitro Cytotoxicity Studies

3.4.1.1. Microscopic Examination

HaCaT cell line used in cytotoxicity studies are immortalized human skin keratinocytes. These cells are anchorage-dependent that express essentially all epidermal differentiation markers. When grown as a monolayer in tissue culture polystyrene dishes (TCPS), they gained polygonal shape (Figure 3.17).

3T3 fibroblast cell line used in cytotoxicity studies are immortalized mouse skin fibroblasts. They are the major cell type of the dermis, and responsible for producing and maintaining most of the extracellular matrix, mainly collagen. Therefore, these cells play a critical role in wound healing. Fibroblasts are morphologically heterogeneous with diverse appearances depending on their location and activity (http://en.wikipedia.org/wiki/Fibroblast). In this study, the fibroblasts had an elongated spindle-like morphology on TCPS (Figure 3.17). In comparison, fibroblasts are bigger cells than keratinocytes (Figures 3.17-3.18). Also, the cell counting studies showed that the number of keratinocytes was approximately ten times of the fibroblasts on the same surface area.

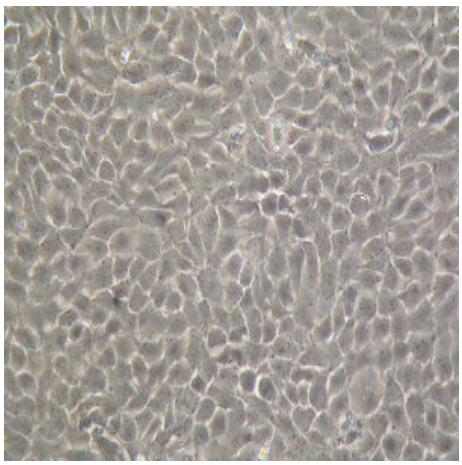


Figure 3.17. Light micrograph of keratinocytes (20X magnification).

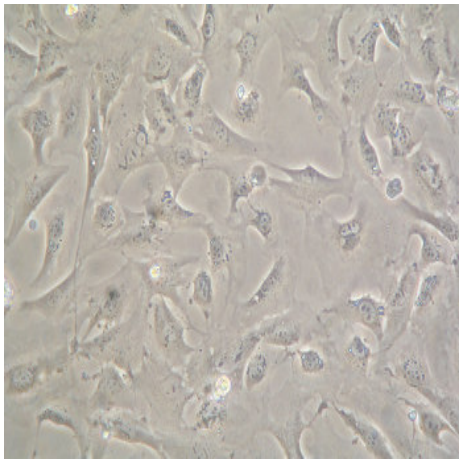


Figure 3.18. Light micrograph of 3T3 fibroblasts (20X magnification)

The morphology of fibroblasts and keratinocytes was examined using light microscopy. Membranes containing sericin had relatively poor vision since sericin inclusion in membranes formed a grainy surface appearance. Light microscopy examinations at 7 day showed that the keratinocyte and fibroblast cells were able to adhere, change morphologically and proliferate on membranes.

Keratinocyte became spread on both thin and thick membranes after 1 day of incubation (Figure 3.19). Microscopic examinations revealed that there was no significant difference among groups of thin membranes after 1 day in terms of cell adhesion. However, thick membranes containing high ratios of collagen (1/2k-x and Ck-x) seemed to contain more cells than other groups at day 1 (2/1k-x, 1/1k-x). For each membrane group, confluency was reached in the middle of the membrane 1 day after seeding and the membrane area covered by cells increased visibly at the end of 7 days. The high proliferation rate of keratinocytes could be explained with the fact that surface properties of membranes were found suitable by these cells. At the end of 7 days, the keratinocytes covered almost all the surface area of the thin membranes. There was no visible distinction between the groups of thin membranes. The cells covered a smaller area of thick membranes in comparison to thin ones after 7 days. The size of cells diminished as they proliferated radially from the middle of membrane to outwards.

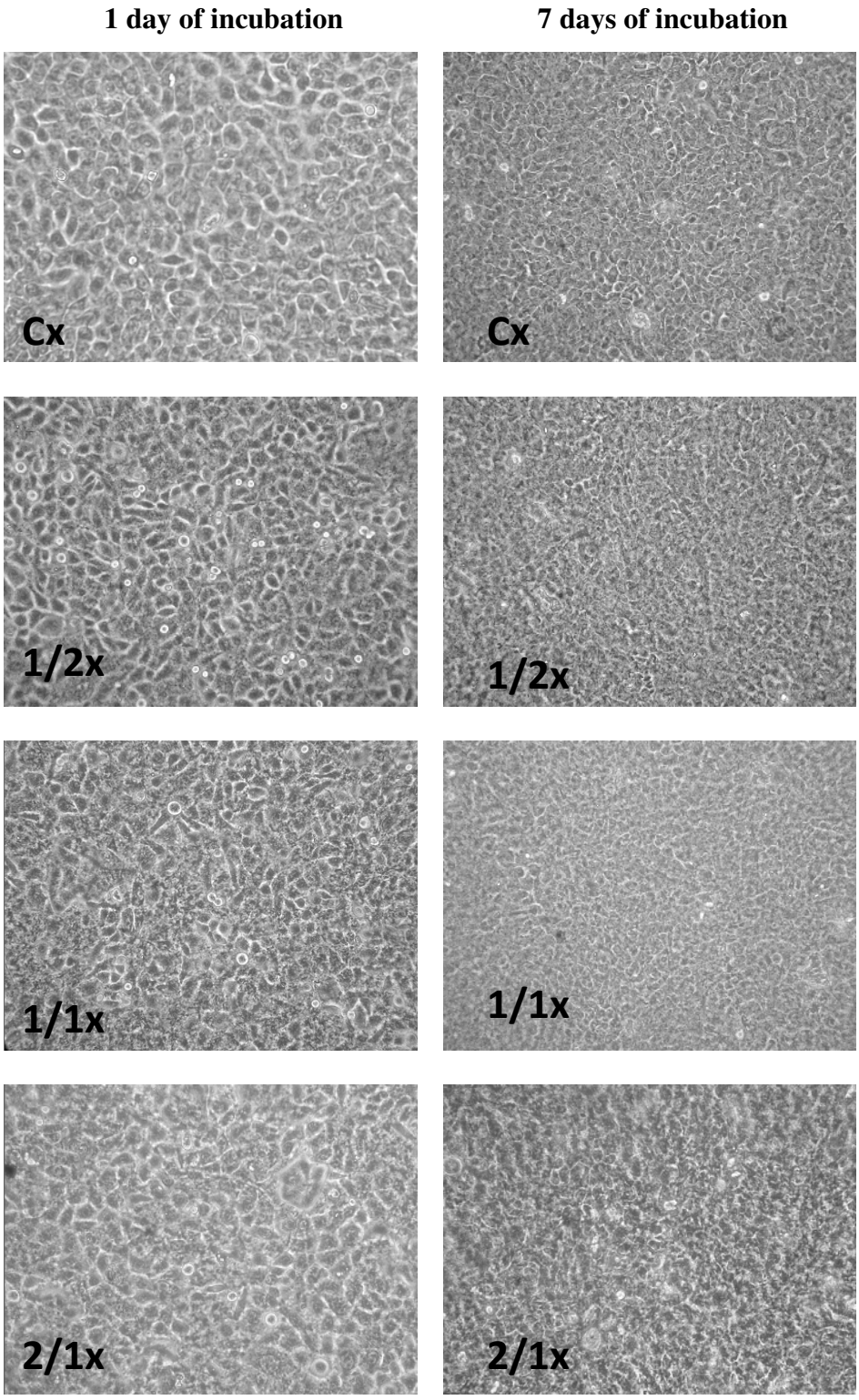


Figure 3.19. Light micrographs of keratinocytes seeded on cross-linked thin membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x) (20x).

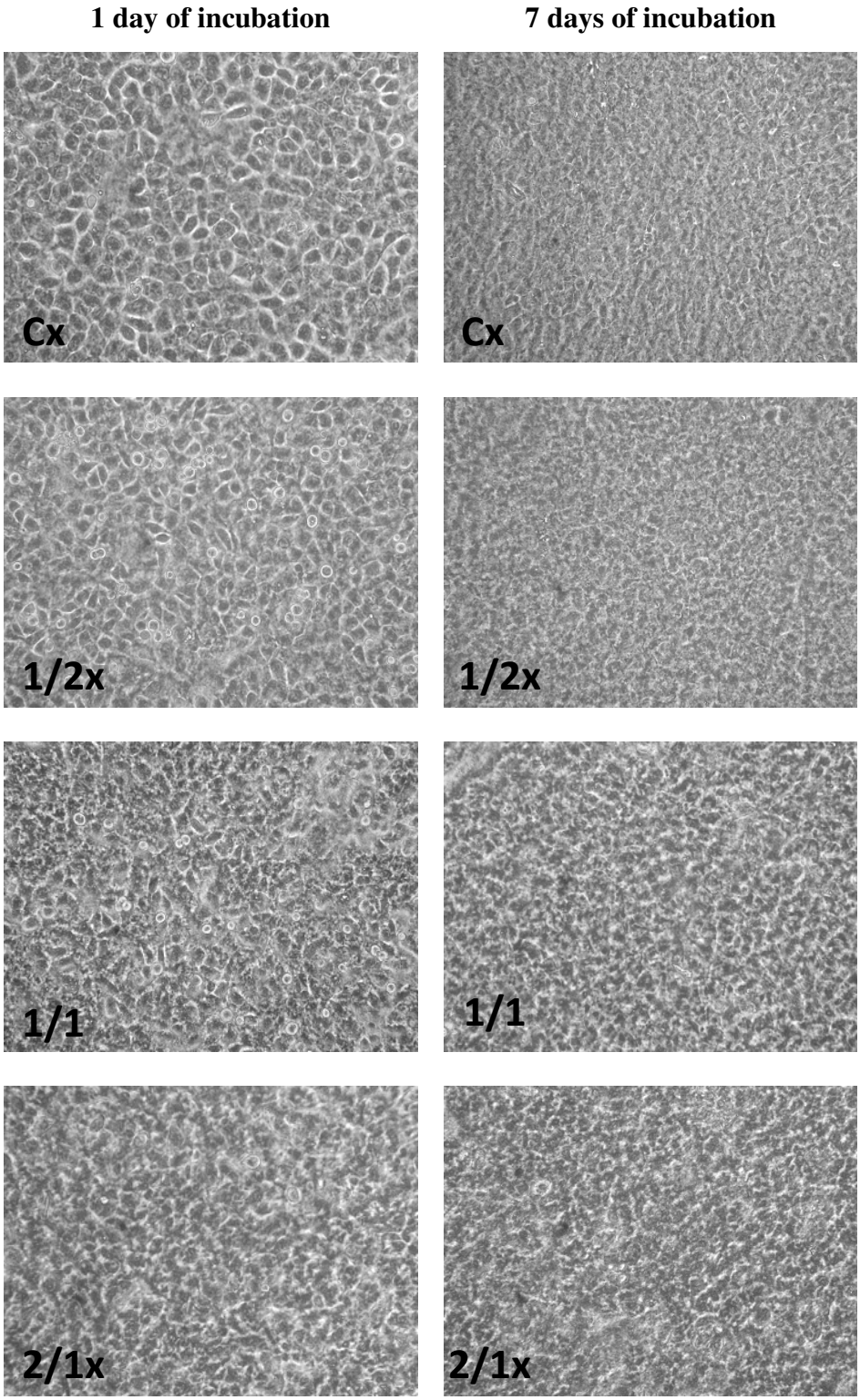


Figure 3.20. Light micrographs of keratinocytes seeded on cross-linked thick membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x) (20X).

Cells attached, proliferated and reached confluency at the end of 7 days. However, after reaching confluency, keratinocytes began to form a stratified structure (Figure 3.21).

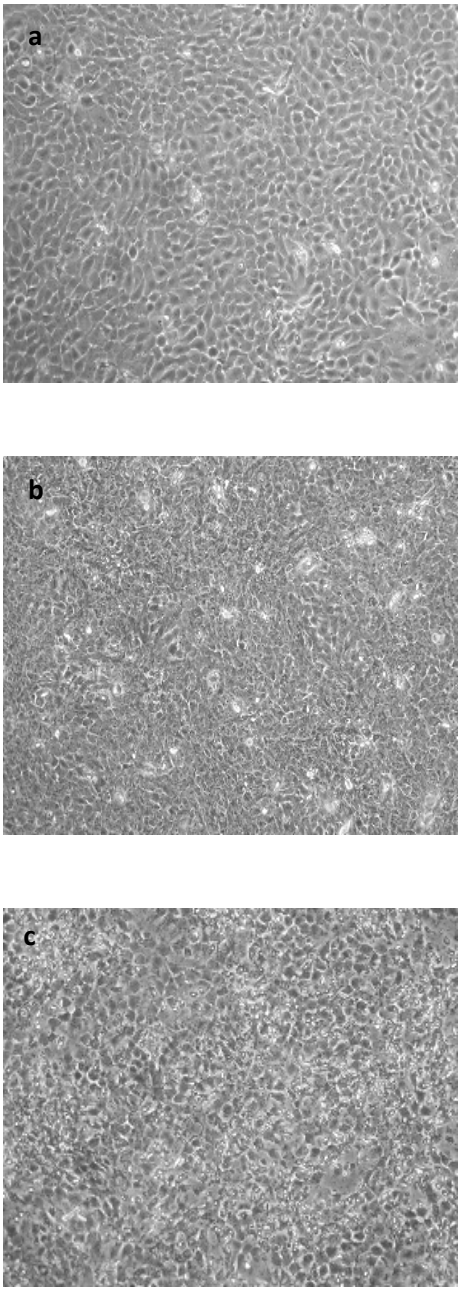


Figure 3.21. Light micrographs of keratinocytes grown as (a) monolayer, (b) and (c) multilayer

3T3 fibroblasts were attached and spread on each group of thin and thick membranes after 1 day (Figure 3.22). It was shown that cells grown on collagen adopt an elongated morphology and were observed to have large pseudopodia-like processes extending onto collagen matrix (Elsdale and Bard, 1972). Microscopic investigations showed that the adherence and proliferation of 3T3 fibroblasts was higher on Ci-x, 1/2i-x, 1/1i-x in comparison to that observed on 2/1i-x membranes. This difference became more evident after 7 days (Figure 3.22). The cell spreading was similar for Ci-x, 1/2i-x, 1/1i-x groups after 7 days. The percentage of spread cells on Ck-x was higher than the percentage observed on other groups of thick membranes. Generally, the fibroblasts got bigger and lost their elongated morphology in areas of membranes where they reached high confluence after 7 days. The cells seeded on groups containing high ratios of sericin (2/1k-x, 1/1k-x) could not be detected easily after 7 days. Therefore, these visual microscopic observations needed verification with further analysis such as SEM and MTT. Yet, the cells on pure collagen membranes were quite confluent and easily detectable.

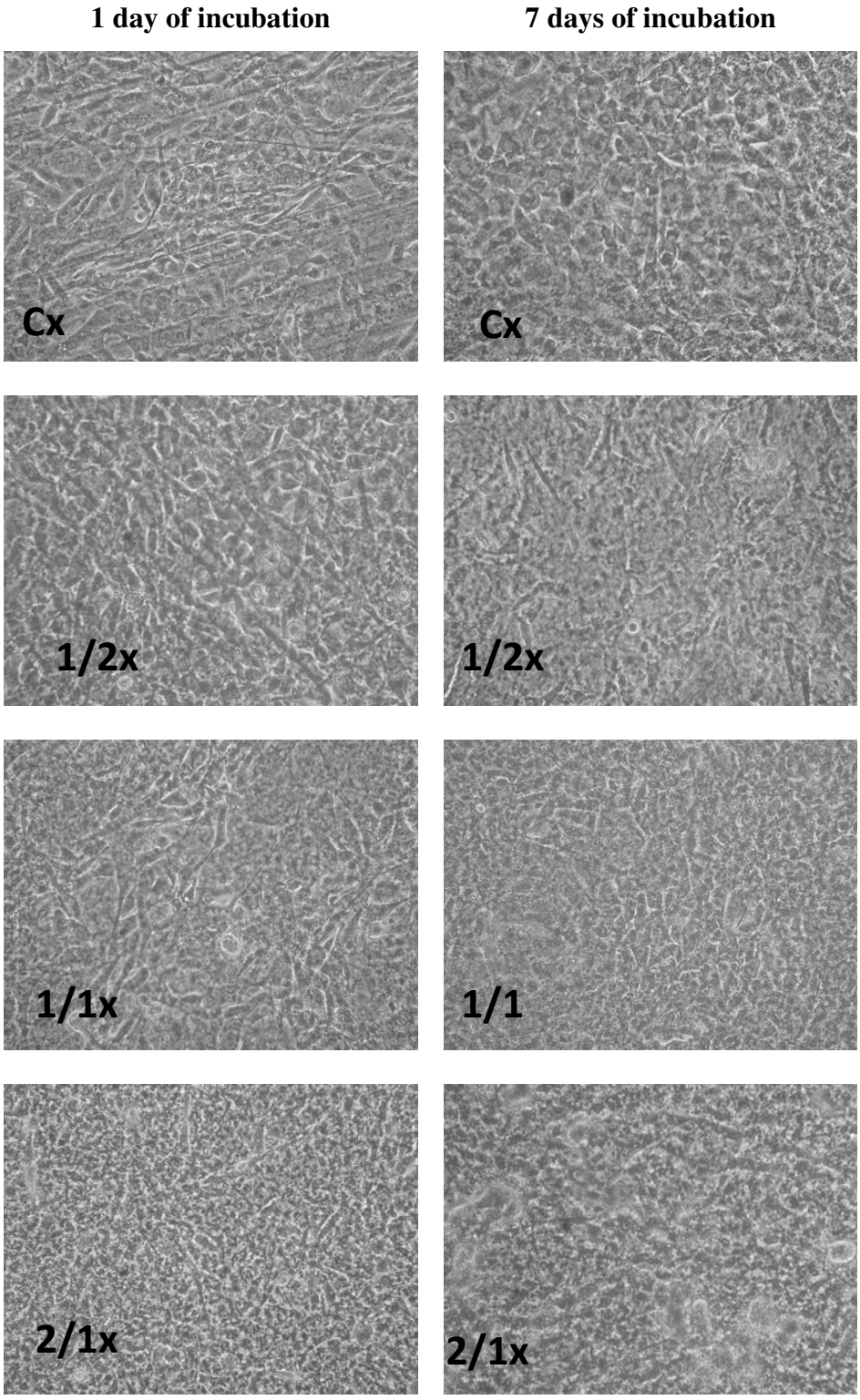


Figure 3.22. Light micrographs of 3T3 fibroblasts seeded on cross-linked thin membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x) (20x).

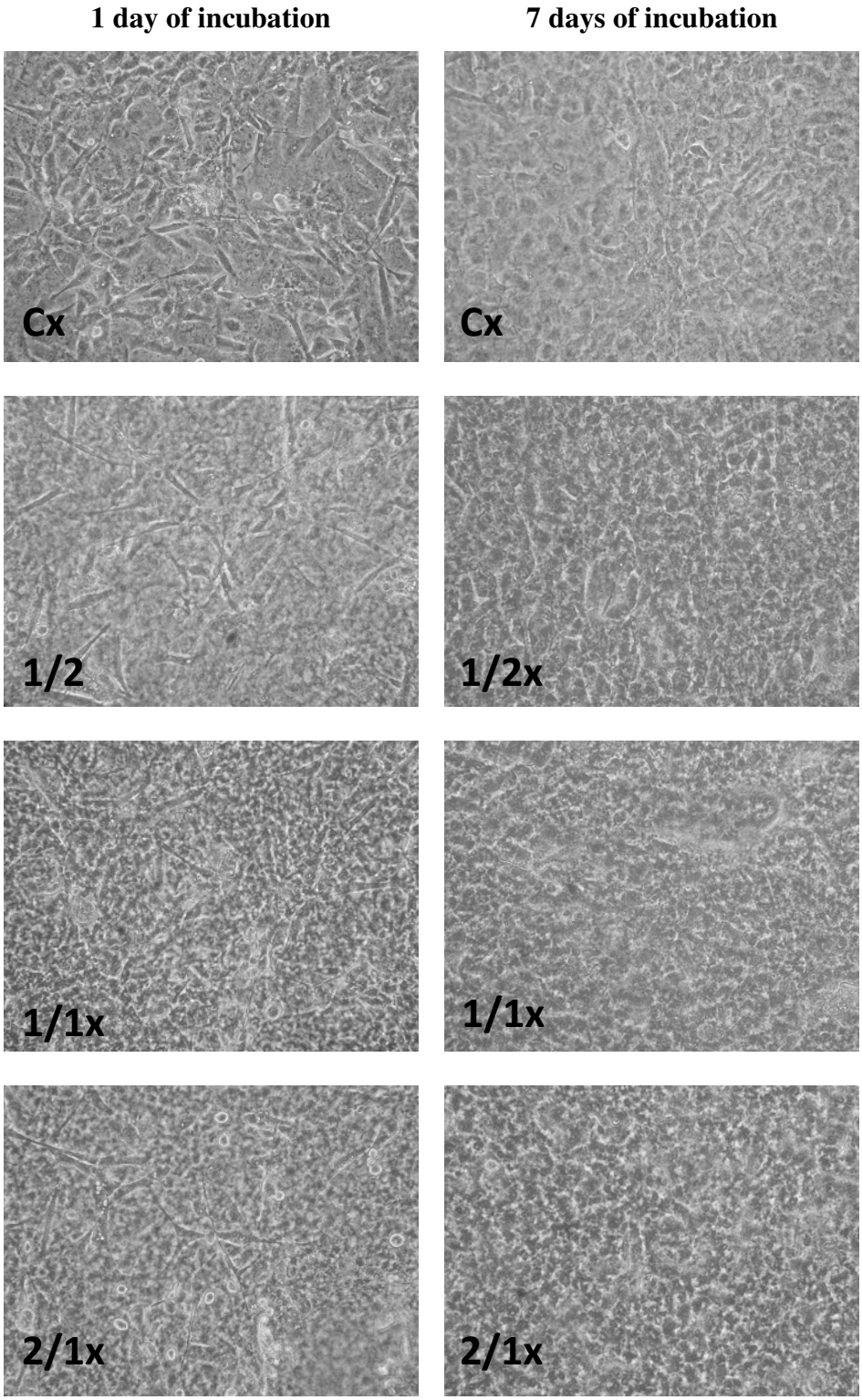


Figure 3.23. Light micrographs of 3T3 fibroblasts seeded on cross-linked thick membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x) (20x).

3.4.1.2. Proliferation Studies

Keratinocytes were more prone to attach to the surfaces of membranes after 1 day incubation compared to positive controls. It is known that collagen is a natural substrate for cellular attachment, growth and differentiation in its native state because certain sequences of the collagen fibrils are chemotactic and promote cellular proliferation and differentiation (Ruszczak, 2003). Therefore, collagen based substrates were used as cultured skin substitutes consisting of keratinocytes and/or fibroblasts (Boyce et al., 1988; Boyce et al., 1995;Yamada et al., 1999; Yannas et al., 1989; Bell et al., 1981). Cell-biomaterial surface interaction occurs via cell surface receptor binding with adhesion ligands on the biomaterial. A high collagen ratio in membranes could be expected to promote the cell attachment via integrin binding sites of collagen. Likewise, it was reported that high collagen content would be expected to promote cell attachment due to integrin binding with cell adhesion domains in collagen such as the RGD sequence (Hubble et al., 1995; LeBarron et al., 2000).

A significant increase in the activity of keratinocytes was observed at day 4 in comparison to day 1 for the positive control. The cells reached to near confluency after 4 days of incubation and therefore, they did not increase significantly in number (Figure 3.24). There were significant increases in cellular activities of keratinocytes with incubation time for all groups of thin membranes, except for 1/2i-x. However, cells proliferated on 1/2i-x significantly after 4 days of incubation and reached to a highly confluent state after 7 days. As a whole, the cells on each thin membrane proliferated significantly with time. After 4 days, the degree of proliferation on Ci-x was comparable to that observed on control. The MTT reading for Ci-x was significantly higher than all other groups of thin membranes. After 7 days, there was no statistical difference between any of the groups and the control.

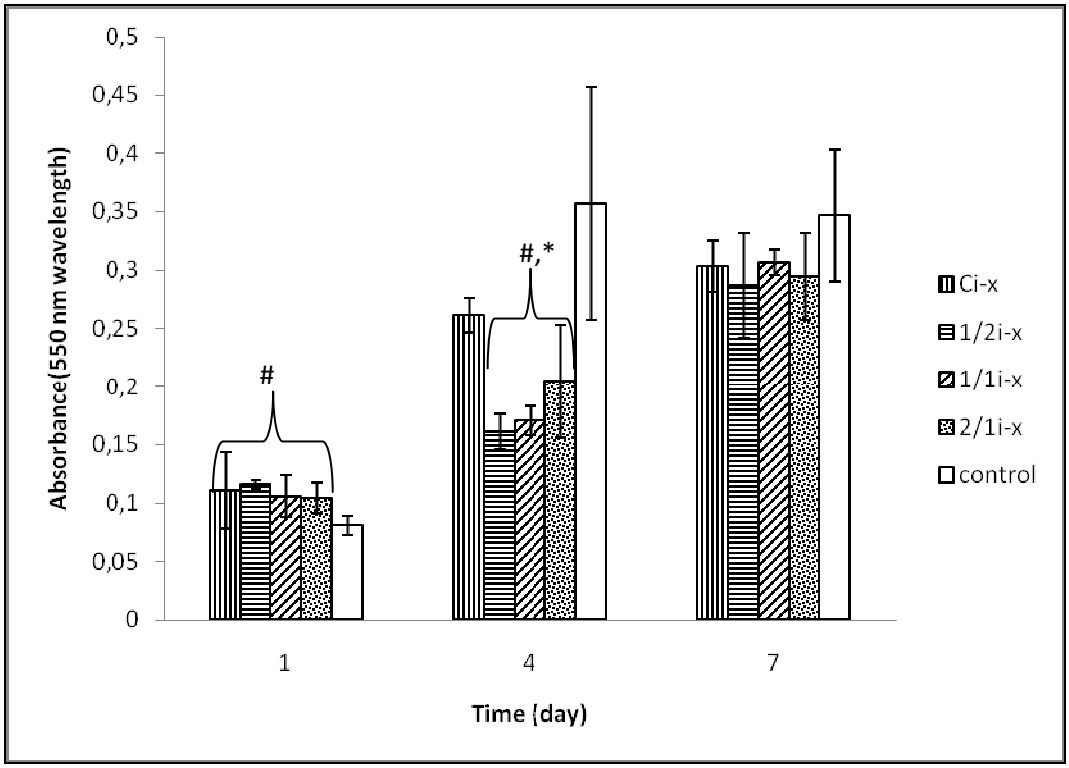


Figure 3.24. Proliferation of keratinocytes on thin membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 4$). $^{\#}$: Significant difference from the control for the same incubation time. *: Significant difference from Ci-x for the same incubation time. $p < 0.05$.

A significant increase in MTT readings from day 1 to 4 was observed in 1/1k-x and 1/2k-x groups implying that cells proliferated on these membranes. However, the cells proliferated significantly on all thick membranes after 4 days. All thick membranes had significantly higher OD values than control on the first day. The cellular activity observed on Ck-x was significantly higher than all other thick membranes. After 4 days, MTT readings on thick membranes were significantly lower than the control. There was no statistical difference between thick membranes for 4 day in vitro culture of keratinocytes. After 7 days none of the groups were statistically different from each other and they were significantly lower than the control.

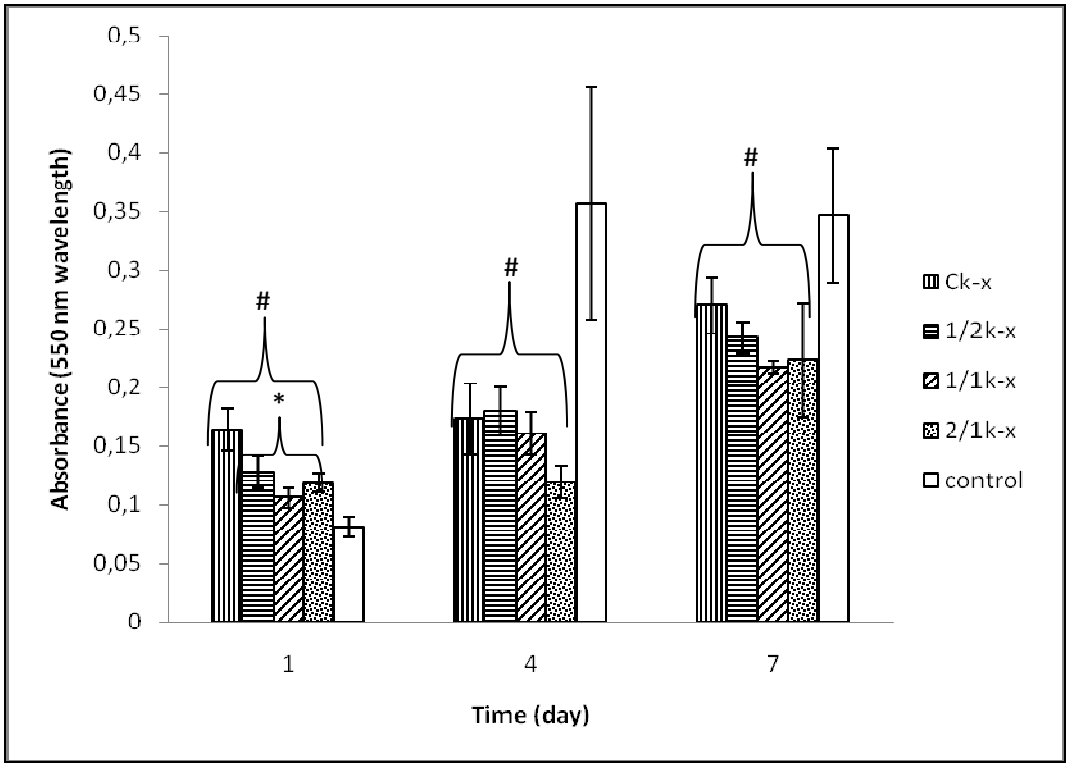


Figure 3.25. Proliferation of keratinocytes on thick membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 4$). $^{\#}$: Significant difference from the control for the same incubation time. *: Significant difference from Ck-x for the same incubation time. $p < 0.05$.

Similar to the keratinocytes, the initial attachment of fibroblasts were high for the same reasons mentioned above. In particular, collagen is present in the extracellular matrix of fibroblasts and these cells link to collagen through α1β1 and α2β1 integrin receptors (Welch et al., 1990; Hynes et al., 1992).

For the positive control results of 3T3 fibroblasts, there was significant increase in cellular activity between 1-4 days and 4-7 days (Figure 3.26). The proliferation of 3T3 fibroblasts on thin membranes was slow up to 4 days, but a significant increase was observed for groups of Ci-x,1/2i-x and 1/1i-x after 4 days of culture. The cellular activities of fibroblasts significantly increased for all thin membrane groups when

cells were cultured for more than 7 days. The cellular activity observed for all thin membranes except for 2/1i- was statistically higher than observed for the control. Also, the OD of 2/1i-x was significantly lower than other groups of thin membranes. The seventh day, all the groups of thin membranes except for 2/1i-x was statistically indifferent than the control and they were significantly higher than 2/1i-x. These results prominently suggest that inclusion of sericin in a ratio of 2:1 resulted with slowing of the proliferation of fibroblasts on thin membranes.

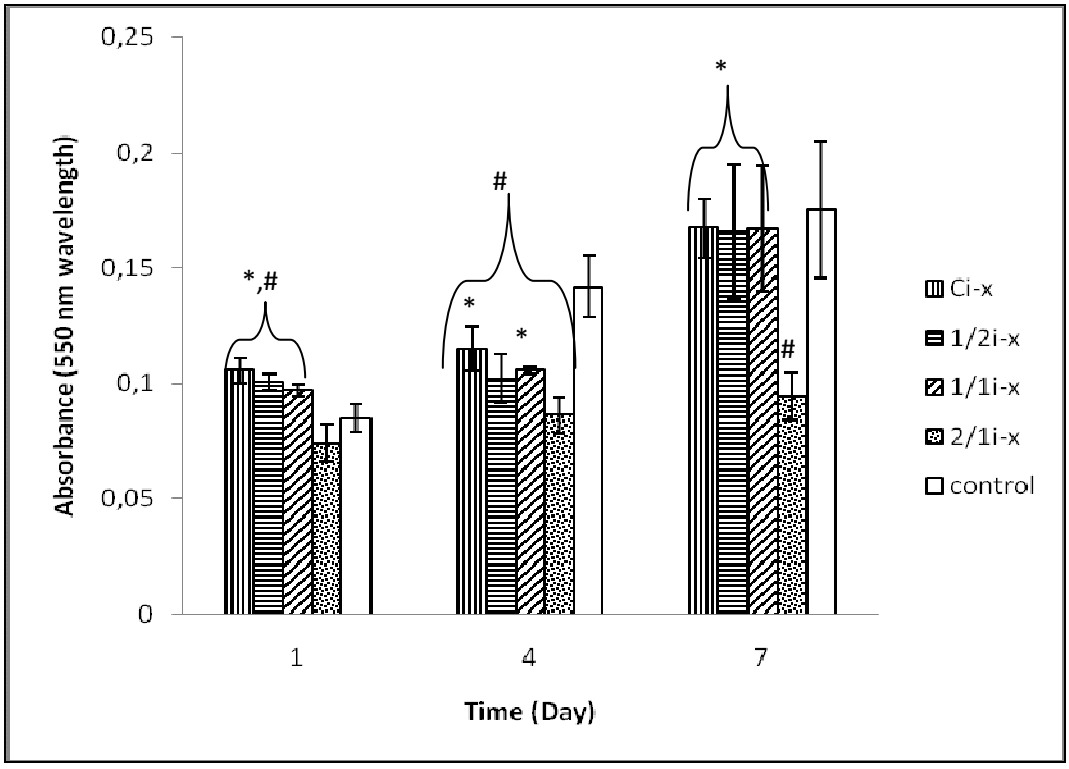


Figure 3.26. Proliferation of fibroblasts on thin membranes containing only collagen (Ci-x), and different weight ratios of sericin/collagen (1/2i-x, 1/1i-x, 2/1i-x). Data are expressed as mean ± SD ($n \geq 4$). $^{\#}$: Significant difference from the control for the same incubation time. *: Significant difference from the group containing the highest sericin ratio (2/1i-x) for the same incubation time. $p < 0.05$.

The cells on Ck-x proliferated significantly (Figure 3.27). The OD increase of 1/1k-x and 2/1k-x membranes between days 1-4 and 1-7 was significant. The MTT readings on Ck-x were significantly higher than the control and all other three groups. At day 4 proliferation of the cells on-2/1k-x was only significantly lower than the control. It was observed that cellular activities on 1/2k-x, 1/1k-x, 2/1k-x were not statistically different from each other. After seven days cells on Ck-x highly proliferated and the OD of this group was higher than all other groups and the control.

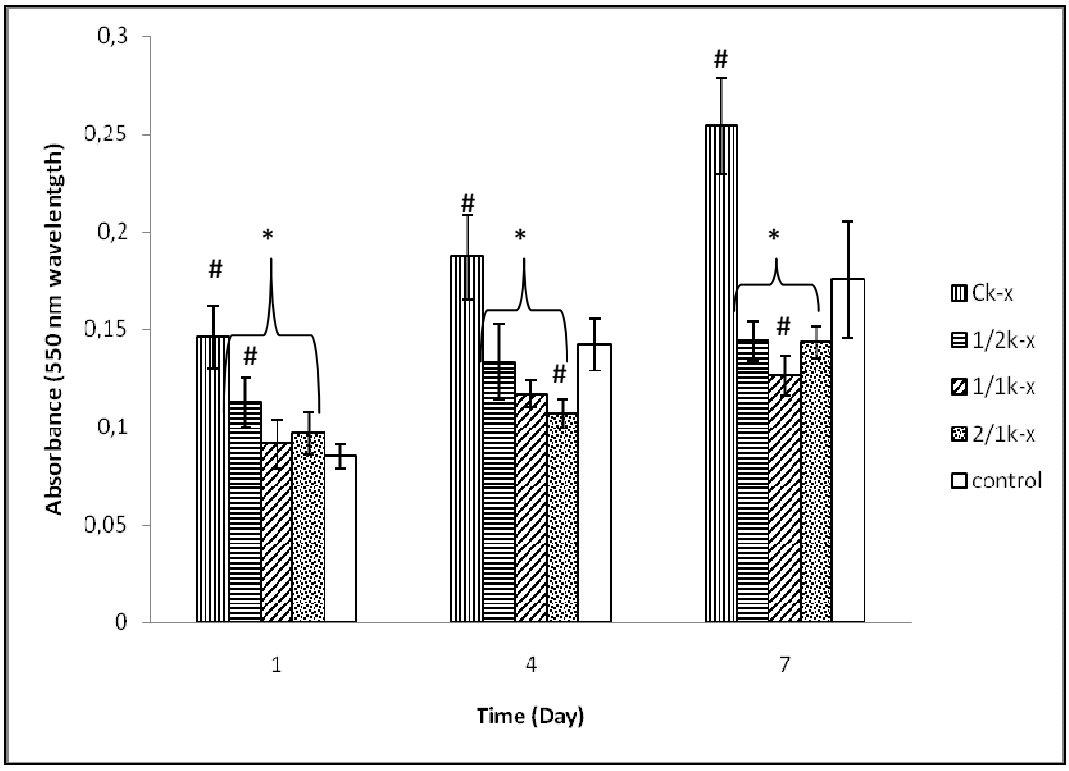


Figure 3.27. Proliferation of fibroblasts on thick membranes containing only collagen (Ck-x), and different weight ratios of sericin/collagen (1/2k-x, 1/1k-x, 2/1k-x). Data are expressed as mean ± SD ($n \geq 4$). $^{\#}$: Significant difference from the control for the same incubation time. *: Significant difference from Ck-x for the same incubation time. $p < 0.05$.

Cell proliferation is not thought to occur within 1 day after seeding. Therefore the high ODs obtained for membranes after 1 day in comparison to controls could be attributed to the enhanced initial cell attachment. Since there was an evident increase in the proliferation of both fibroblasts and keratinocytes on membranes, it could be suggested that the glutaraldehyde cross-linking (3 %) of membranes had no cytotoxic effect. Sheu et al., (2001) studied the cytotoxic effect of GTA at various concentrations on the growth of fibroblasts and demonstrated that there was no sign of cytotoxicity even with a concentration of GTA as high as 0.2 %. The potential cytotoxicity of GTA residues of collagen/chitosan porous scaffolds treated by 0.25% GTA was not evident in the study of Ma et al. (2003). It is suggested that coating a cell culturing surface with type I collagen enhances the attachment and proliferation of keratinocytes. After making a comparison between different cell substrates, Daniels et al. (1997) found out that type I collagen had superiorities in this context but still it did not provide enough evidence to suggest that it might be the best choice for keratinocyte attachment and proliferation. All the groups of thin membranes seemed to be statistically similar with the control after 7 days in terms of proliferation; therefore, it could not be proposed evidently that they were better substrates for keratinocytes. There was no statistically meaningful difference between the proliferations of keratinocytes on any membrane. Dash et al (2008) evaluated the effect of sericin on viability of HaCaT cells by MTT assay and found that cell proliferation was not inhibited at low concentrations of sericin. On the other hand, the membranes containing higher sericin ratios were not favorable surfaces for fibroblasts in comparison to membranes containing only collagen. Furthermore the thick membrane containing only collagen was even better than the control statistically. The results of this study support that cross-linked sericin/collagen membranes could be efficient substrates for cell proliferation, because the results were comparable with the control surface. The recent studies performed on the effect of sericin for the attachment and growth of cells had shown that sericin enhanced the attachment of primary cultured human skin fibroblasts (Tsubouchu et al., 2005). It was proposed as a mitogenic factor for mammalian cell lines (Terada et al, 2002; Terada et al., 2005), and improved islet culture (Agowa et al., 2004).

Cell-cell coupling stemmed from high seeding density on the membrane was likely to be the cause that restricted the complete cell confluency throughout the entire membrane surface. As demonstrated in the Figure 3.28, there were considerably dense keratinocyte colonies in the middle of each membrane type after 7 days of incubation. Keratinocyte multilayers also infiltrated from membrane surface to the culture flask. Consequently, it could be asserted that sericin/collagen membranes were favorable substrates for keratinocyte cell culturing. Keratinocytes were dyed with MTT bromide and the dark brown colour formed on membranes was pictured as a strong evidence of viable keratinocytes on membranes (Figure 3.28).

3.4.1.3. Scanning Electron Microscopy (SEM) Analysis

SEM analysis showed that the surfaces of all membranes were very smooth (Figure 3.29). Upon seeding cells, surface morphology changed and cell boundaries were evident (Figure 3.30).

As analogous to light microscopy analysis, the polygonal morphology of keratinocytes was again observed by SEM (Figures 3.30-3.31). Nonetheless, a layered structure was still manifest. At the end of 7 days, the high degree of conformation of keratinocytes with the surface contours of the membranes apparently indicated effective cell-substrate binding (Dai et al., 2004).

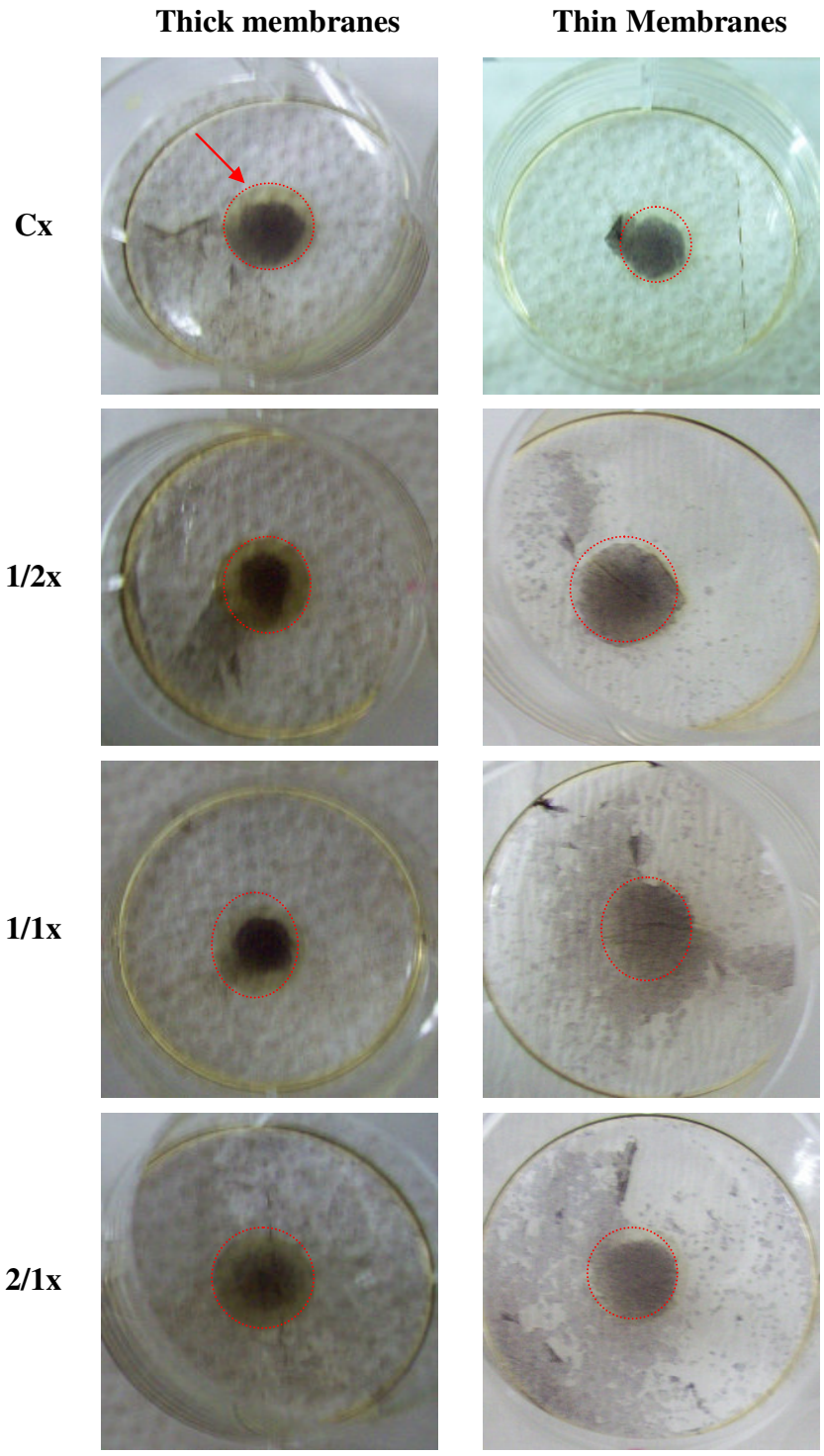


Figure 3.28. Keratinocyte dying of membranes with MTT bromide after 7 days. The red circle is the area of membrane. The groups are cross-linked membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x).

Thin membranes **Thick membranes**

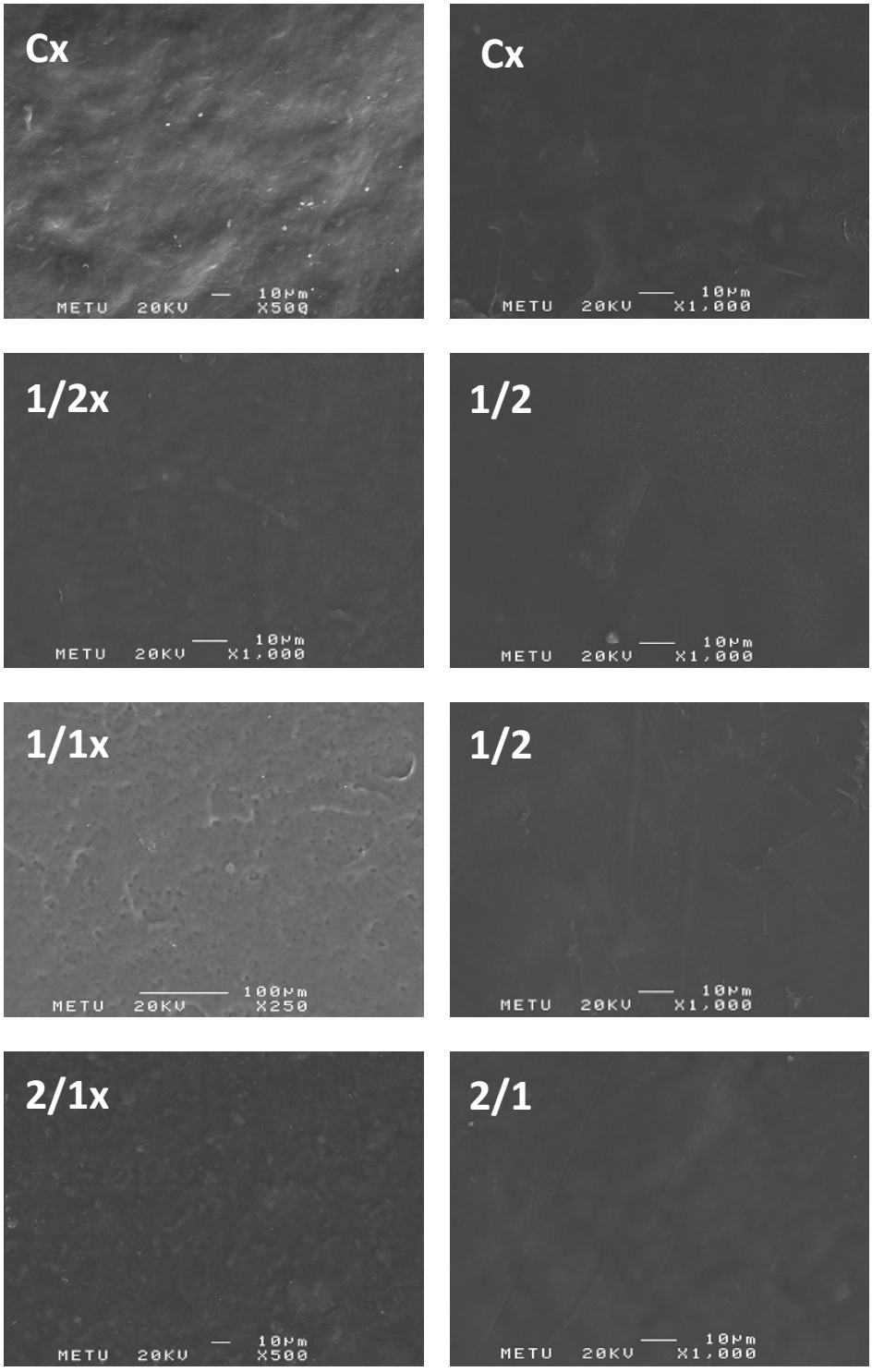


Figure 3.29. SEM images of membranes without cells. The groups are cross-linked membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x).

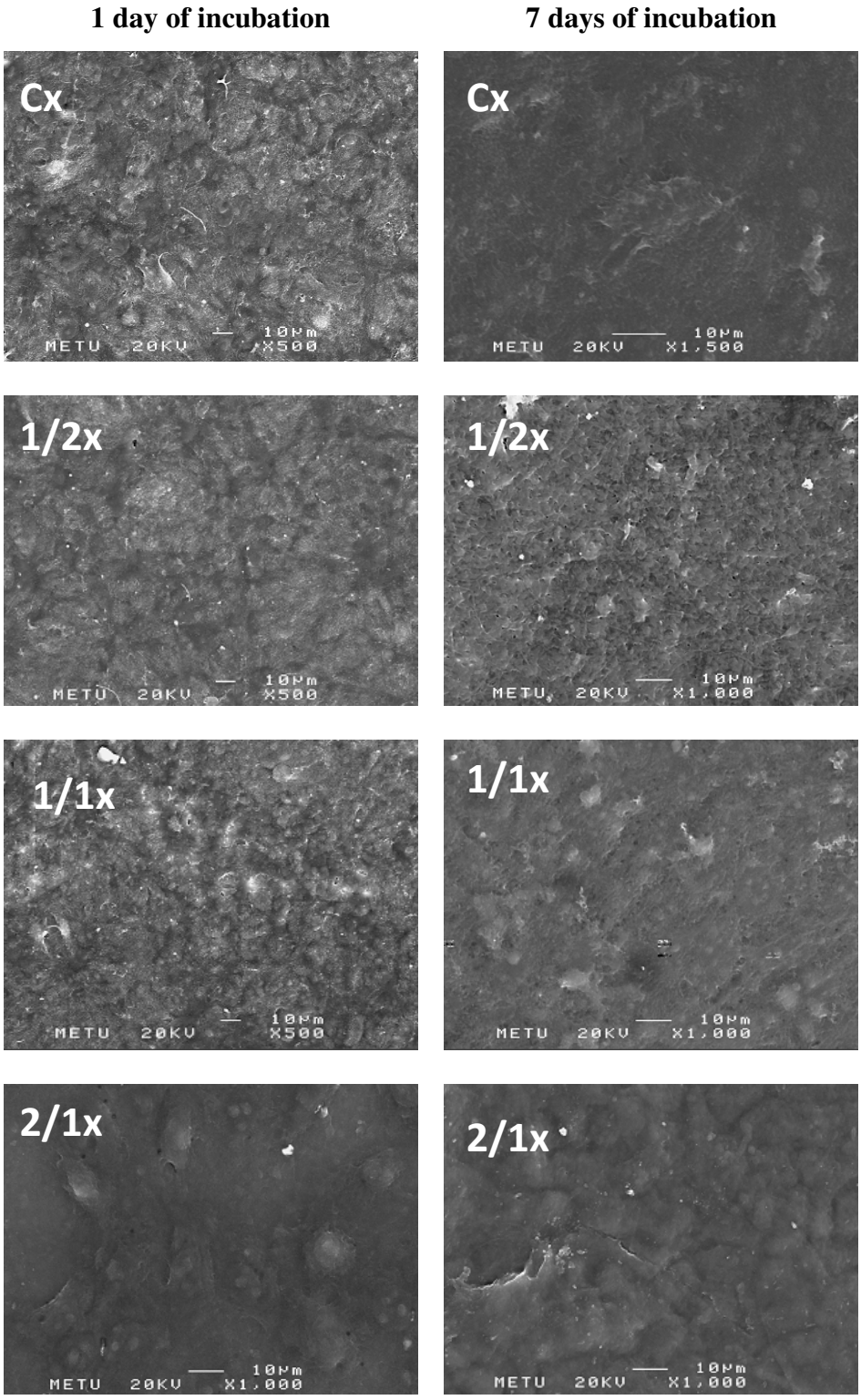


Figure 3.30. SEM images of keratinocytes seeded on cross-linked thin membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x).

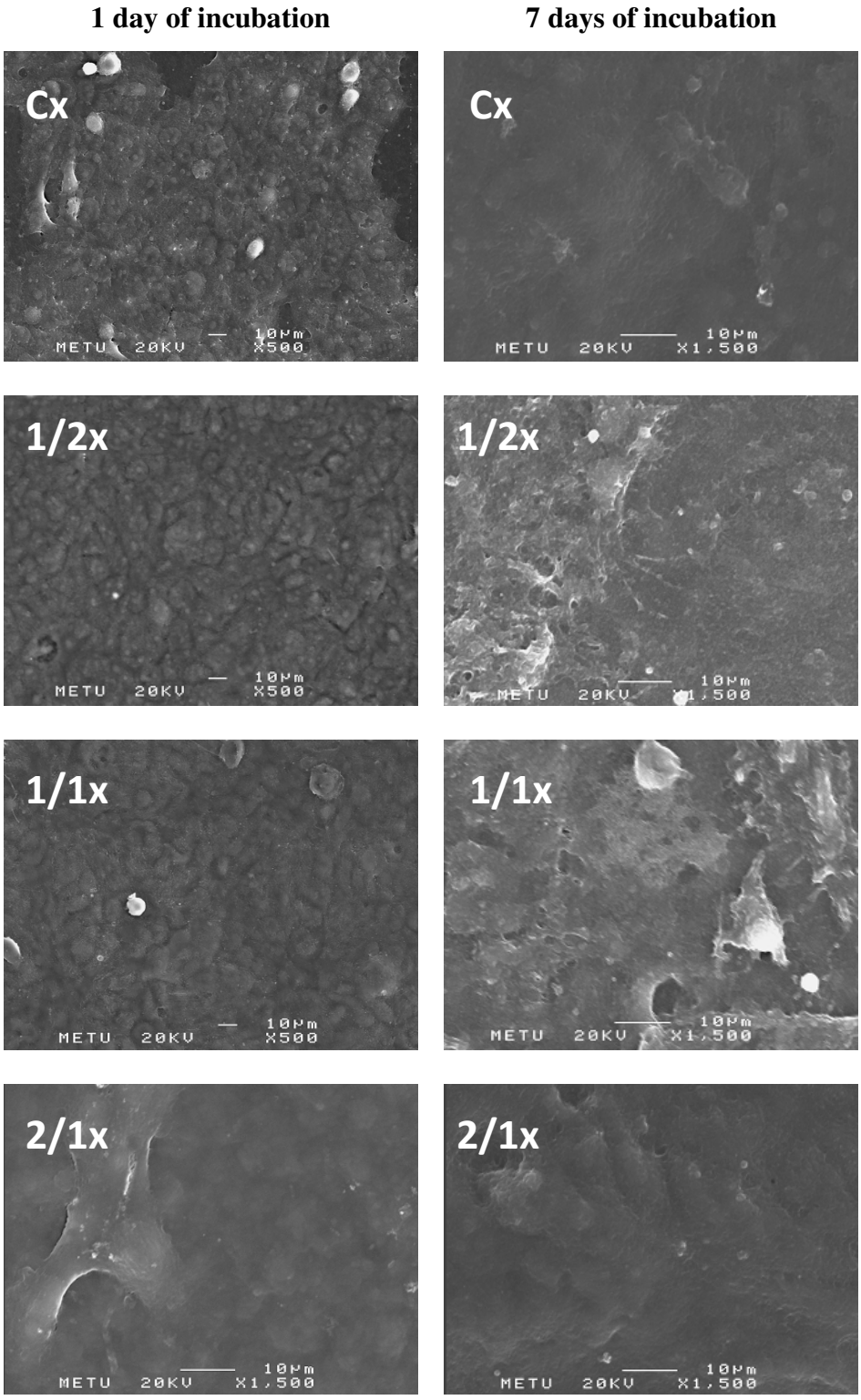


Figure 3.31. SEM images of keratinocytes seeded on cross-linked thick membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x).

As verification to light microscopy observations, fibroblasts gained an elongated morphology on all membranes 1 day after seeding and the attached cells continued to spread out on the membranes during 7 days (Figure 3.32-3.33). 3T3 cells approached to senescent state at high confluency; therefore, they gained larger morphology at the 7^{th} day. The filopadia of fibroblasts were seen in the SEM pictures, indicating that they were strongly attached on the surface. Considering the well extented fibrous cell morphology in this study, sericin/collagen membranes might be suggested as useful substratum for the cultivation of human fibroblasts. Tsubouchi et al. (2005) correlated the attachment ability of cells with different fractions of sericin and thought that serine-rich repetitive domains in sericin might be responsible for attachment. In addition, Minoura et al. (1995) evaluated the attachment and growth of L-929 cells on films made of Bombyx mori silk proteins (fibroin, sericin and their mixtures) and concluded that the phase-separated structure of fibroin and sericin is influential on cell attachment and growth. The phase-separated structure of collagen-sericin membranes might have behaved in a similar manner. Minoura et al. (1995) also stated that the cells attached to sericin had a different shape in contrast to spindle shape observed for collagen and silk fibroin. Therefore, the spindle shape of fibroblasts occurred on sericin/collagen membranes were thought to be due to collagen.

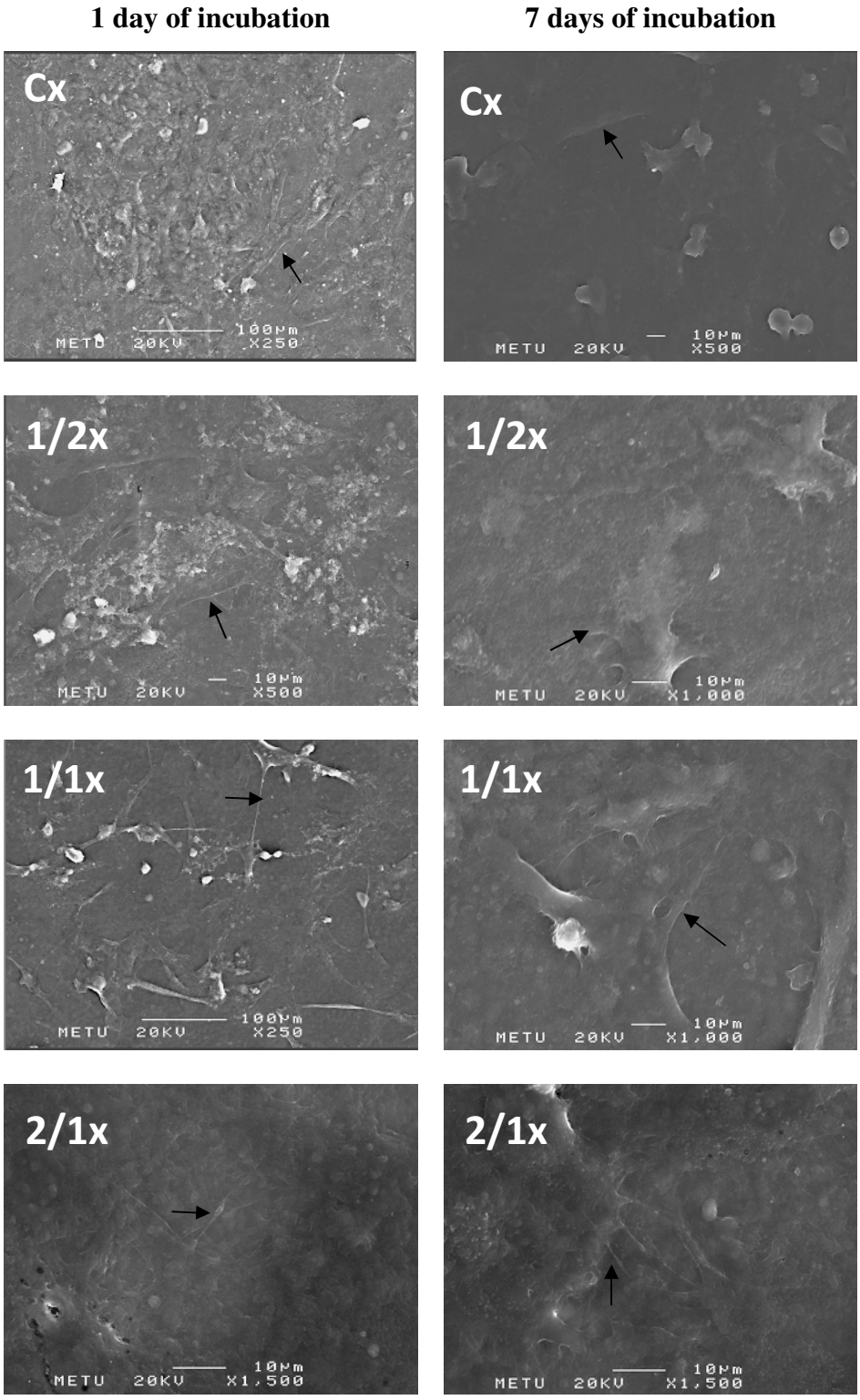


Figure 3.32. SEM images of fibroblasts seeded on cross-linked thin membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x).The arrows indicate filopodias.

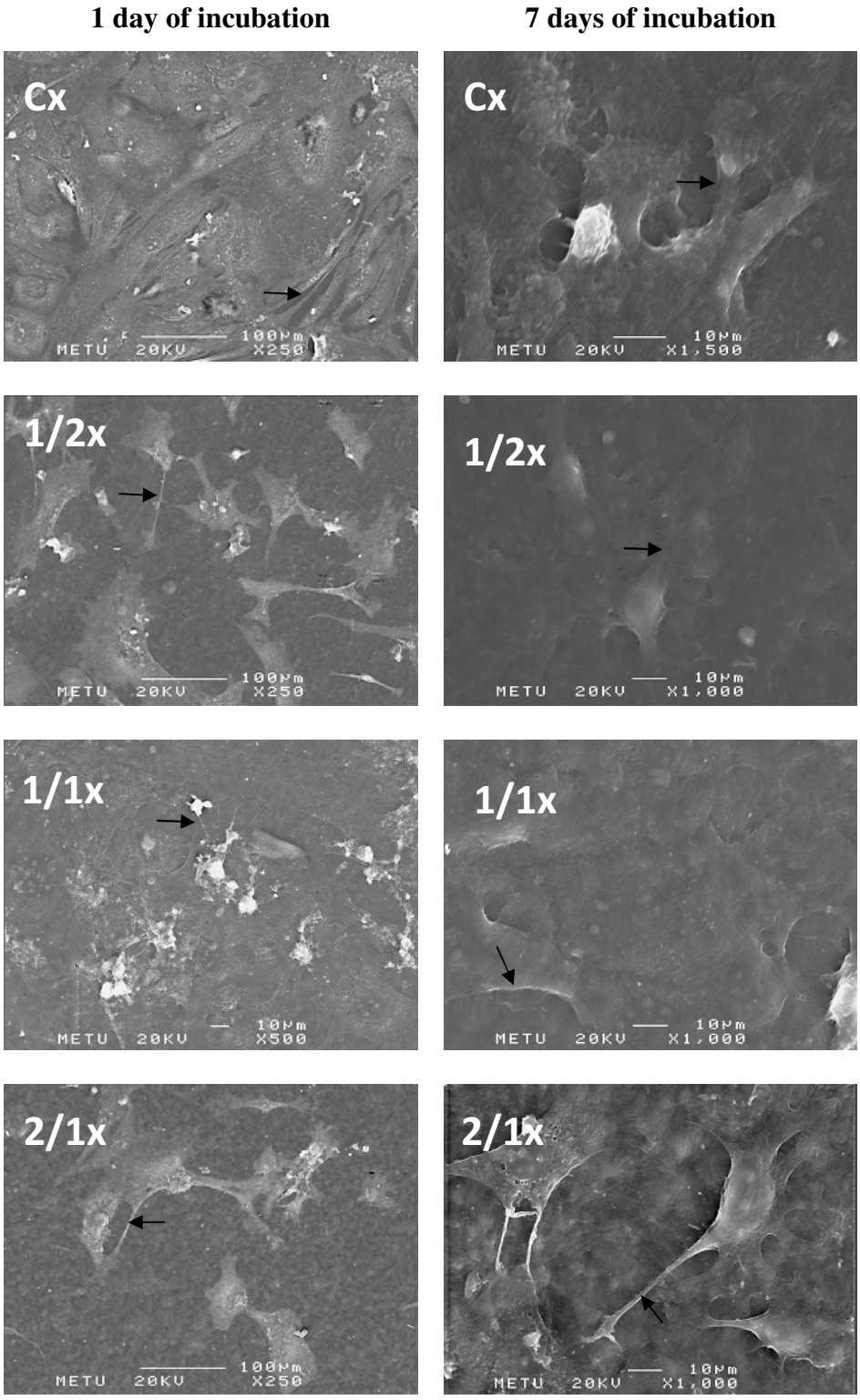


Figure 3.33. SEM images of fibroblasts seeded on cross-linked thick membranes containing only collagen (Cx), and different weight ratios of sericin/collagen (1/2x, 1/1x, 2/1x). The arrows indicate filopodias.

CHAPTER 4

CONCLUSION

Skin is a vital organ that needs treatment with medications and/or a biomaterial if it is damaged with moderate to severe wounds. Many different wound dressing products have been produced for this purpose. Among these biomaterials, the biological dressings attracted great attention of researchers since they have enhanced properties for healing. Hence, in this study collagen, a natural matrix protein and sericin, a structural protein found in silk were used to prepare wound dressing membranes. It was aimed to idealize the membranes in terms of wound dressing properties by manipulating the composition and thickness of membranes.

Membranes were also cross-linked with GTA (3 % w/v) to increase their resistance to hydrolytic degradation and to improve their mechanical properties. Uncross-linked groups lost their structural integrity before 2 weeks, whereas cross-linked membranes were highly resistant to hydrolytic degradation and they only lost 10.27- 23.37 % of their weights during 4 weeks. Degradation of membranes containing sericin was high than the groups containing collagen only.

The EDS of cross-linked membranes had a range of 14.91 to 4.37 (g/g). This range was thought to be comparable with similar wound dressing materials in literature. Sericin was known to be a hydrophilic protein and have good moisture absorbing and desorbing properties. Parallel to these, the increase in sericin ratio of membranes significantly increased the EDS of membranes up to a particular ratio, but further increase resulted with a decrease. The highest EDS was obtained with 1/1 (sericin/collagen) membranes.

No obvious relationship could be found between the ratio of proteins and water vapor transmission rate (WVTR). However, when the thickness of membrane was

increased, the WVTR of membranes dropped significantly. In general, the WVTR range of membranes (980.90-1084.03 g/m^2/day) was found suitable for low to medium exudating wounds.

All groups of membranes were permeable to oxygen. Membrane thickness and sericin proportion affected the oxygen permeability. Accordingly, thin membranes containing sericin had significantly enhanced oxygen permeability.

All the membranes were found to have mechanically good properties in comparison to other wound dressing materials in the literature. The range of UTS was 10- 45 MPa. As sericin ratio was increased UTS decreased. EB of dry membranes reached its maximum value of 13 % with only collagen membranes and diminished to its lowest value of 2 % with the highest sericin proportion. E values of membranes were in agreement with the literature. As a whole, sericin decreased the membrane mechanical properties such as tensile strength and elongation compared to only collagen membranes

Membranes were found to be superior for their ability to prevent the passage of exogenous microorganisms. Thus, they were able to eliminate secondary infections. This result could be attributed to the dense nonporous structure of membranes and to the antibacterial property of sericin.

Biocompatibility of membranes was investigated on skin cells, 3T3 fibroblasts and HaCaT keratinocytes. Response of seeded cells to each membrane type was evaluated by MTT cell viability assays and by microscopy. The initial attachment of both keratinocytes and fibroblasts were significantly higher than the control. Keratinocytes proliferated significantly on each membrane during 1 week and reached to high confluency compared to positive control. Similarly, fibroblasts also proliferated significantly on all membranes except for the membranes containing the highest sericin ratio. Light microscopy and SEM analysis showed that both cells could attach and spread on membranes. Also, both cells gained their characteristic

morphology on membranes and flattened within 7 days. Hence no cytotoxic effect was observed in any groups of cross-linked membranes.

Eventually, it could be concluded that sericin-collagen membranes are very promising candidates for wound dressing applications. Their properties could be tailored by changing their proportions and thicknesses. Among the groups produced in this study, the sericin/collagen membranes in 1/1 and 1/2 proportions had better wound dressing properties. Their thicknesses could be adjusted regarding to effect of thickness on oxygen permeability and WVTR. For a final conclusion on usability of these membranes, *in vivo* applications should be carried out. In addition, if they are produced in a form which mimics more the structure of skin, they might give even better results. It could be proposed that producing a skin construct consisting of collagen sponge lower layer (as a dermal substitute) and a sericin-collagen membrane upper layer (as an epidermal substitute) could provide all the requirements for an ideal wound dressing.

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