A STUDY OF POROUS SILK FIBROIN-PVA HYDROGEL BY GAMMA IRRADIATION FOR ARTIFICIAL SKIN SUBSTITUTE

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIRMENTS FOR
THE DEGREE OF MASTER OF ENGINEERING
(INTEGRATED CHEMICAL ENGINEERING)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2011

Thesis
entitled

A STUDY OF POROUS SILK FIBROIN-PVA HYDROGEL BY GAMMA IRRADIATION FOR ARTIFITIAL SKIN SUBSTITUTES

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was submitted to the Faculty of Graduated Studies, Mahidol University for the degree of Master of Engineering (Integrated Chemical Engineering)
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ACKHOWLEDGEMENTS

The success of this thesis can be succeeded by supporting from Thai atomic office and Thailand institute of nuclear technology, these two institutes are greatly kindness to convenient a place for doing research. Especially, Mrs, Harined Mungpayabal and Mr, Amornthrep Kojung that are suggesting and teaching my thesis.

Thanks you for every advisor that advice me everything about my thesis, Ass. Prof. Bovornlak Oonkhanond, Ass. Prof. Threerapron Rubcumintara

Finally, I would like to apologize that I did wrong before and I promise to improve myself.

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ABSTRACT

The artificial skin substitute is fabricated by blending PVA in silk fibroin solution at varied ratios. The high content of PVA causes the sol-gel transformation time of hygdrogel to be shorter. A silk fibroin composite solution with PVA was gelled using gamma irradiation. The result shows that the higher irradiation dose makes gelation time faster than using lower doses. The maximum degree of swelling occurs at 25 kGy. The ATR-FT-IR shows a transformation of sol-gel transition between random-coil to beta-sheet structure at amide I (1700-1600 cm^{-1}). Therefore, structures of silk fibroin peak band are changed by gamma irradiation from 1650 to 1625 cm^{-1} peak area. This transformation of silk fibroin structure is achieved at 40 kGy. The peak of random-coil (1650 cm^{-1}) disappears and then the peak of beta-sheet can be seen clearly. The result of *in vitro* degradation found that the hydrogel irradiated by lower doses degrades faster than the hydrogel irradiated by higher dose and the high content amount of PVA caused it to degrade more slowly. A concentrated condition of enzymatic degradation indicated that using 1, 0.1, and 0.01 mg/ml concentrations of protease enzyme displays that higher concentrations can degrade very quickly. The Porosity of silk-PVA hydrogel was scanned and showed in the hydrogel structure that the added salt particles were at varying ratios. The hydrogel with a higher salt particle ratio showed a high amount of pores. Cyto-toxicity and cell culture were tested with silk-PVA hydrogel. The results of testing showed that fibroblast cells can grow, proliferate, and attach within the hydrogel. Results indicated that this silk-PVA hydrogel can support a biocompatibility and application as an artificial skin substitute.

KEY WORDS: SILK FIBROIN/ GAMMA-IRRADIATION/ ARTIFICIAL SKIN SUBSTITUTE / SILK FIBROIN-PVA HYDROGEL

65 pages

การศึกษาไหมไฟโบรอีน-โพลีไวนีลแอลกอฮอลล์ไฮโดรเจลที่มีรูพรุนโดยการฉายรังสีแกมม่าสำหรับวัสดุเทียมทดแทนผิวหนัง

A STUDY OF POROUS SILK FIBROIN-PVA HYDROGEL BY GAMMA IRRADIATION FOR ARTIFICIAL SKIN SUBSTITUTE

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บทคัดย่อ

วัสดุเทียมทดแทนผิวหนังถูกสร้างขึ้นโดยการผสมโพลีไวนีลแอกอฮลล์ (PVA) ลงในสาระละลายไหมไฟโบรอีน ณ ระดับปริมาณต่างๆกัน ปริมาณPVAที่สูงเป็นเหตุให้เวลาในการเปลี่ยนแปลงสารละลายเป็นเจลนั้นสั้นลง สารละลายผสมไหมไฟโบรอินPVAถูกทำให้เป็นเจลโดยการฉายรังสีแกมม่า ผลลัพธ์แสดงให้เห็นว่าที่ความเข้มข้นของการฉายรังสีสูงๆนั้นทำให้เวาลในการเกิดเจลนั้นเร็วกว่าการฉายรังสีที่มีความเข้มข้นต่ำ ระดับการบวมน้ำสูงสุดที่เกิดขึ้นคือที่ 25 กิโลเกย์ การวัดการเปลี่ยนแปลงโครงสร้างแบบ ATR-FTIR แสดงให้เห็นถึงการเปลี่ยนแปลงจากสารละลายไปเป็นเจลระหว่างโครงสร้างแบบแรนดอมคอยกับเบต้าชีสที่เอมไมด์หนึ่ง (1700-1600 cm^{-1}) ดังนั้นโครงสร้างของตำแหน่งของโมเลกุลไหมไฟโบรอีนถูกเปลี่ยนแปลงโดยการฉายรังสีแกมม่าจาก 1650 ไปที่ 1625 cm-1 การเปลี่ยนแปลงนี้ของโครางสร้างของไหมไฟโบรอีนนี้ถูกทำให้สำเร็จที่ 40 กิโลเกย์ ตำแหน่งของแรนดอมคอย (1650 cm-1) หายไปและตำแหน่งบีต้าชีสได้ถูกเห็นอย่างเด่นชัด

ผลลัพธ์ของการย่อยสลายพบว่าไฮโดรเจลถูกฉายรังสีด้วยความเข้มข้นที่ต่ำมันย่อยสลายได้เร็วกว่าไฮโดรเจลที่ถูกฉายด้วยความเข้มขันที่สูงและปริมาณPVAที่สูงสามารถเป็นผลกระทบต่อเวลาในการย่อยสลายช้าลง เงื่อนไขความเข้มข้นของการย่อยสลายโดยเอ็นไซม์ถูกบ่งชี้ว่าสามารถย่อยสลายการใช้ความเข้มข้นที่ 1, 0.1, 0.01 มมต่อมล ของความเข้มข้นเอ็นไซม์โปรตีเอสแสดงให้เห็นว่าที่ความเข้มข้นสูงสามารถย่อยสลายได้อย่างรวดเร็วตามลำดับ ความเป็นรูพรุนของไหมPVAไฮโดรเจลได้ถูกสแกนและแสดงภายในโครงสร้างไฮโดรเจลที่ถูกใส่อนุภาคเกลือที่อัตราส่วนต่างๆ ไฮโดรเจลที่ถูกเติมด้วยอัตราส่วนของอนุภาคเกลือสูงๆแสดงปริมาณจำนวนรูพรุนที่สูงภายในไฮโดรเจล การเลี้ยงเซลล์และการทดสอบความเป็นพิษถูกทดสอบร่วมกับไหมพีวีเอไฮโดรเจล ผลลัพธ์ของการทดสอบนั้นแสดงว่าไฟโบรบลาสเซลล์สามารถเติบโต แพร่กระจาย และแบ่งจำนวนภายในไฮโดรเจล มันถูกบ่งชี้ว่าไหมPVAไฮโดรเจลนี้สามารถสนับสนุนความเข้ากันได้และการประยุกต์ใช้อย่างวัสดุเทียมทดแทนผิวหนังได้

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LIST OF ABBREVIATIONS

%	percent
^{o}C	degree Celsius
γ-ray	gamma-ray
Aw	water absorption
cm	centimeter
ECM	extracellular matrix
EWC	equilibrium water content
FT-IR	Fourier transforms infrared spectroscopy
GA	Glutaradehyde
GAG	Glycosaminoglycans
Hr	hour
kDa	kilodalton
kGy	kilogray
mL	milliliter
mM	millimolar
mm	millimeter
m_p	mass loss
m_{pi}	The initial dry hydrogel mass
NaOH	sodium chloride
nm	nanomater
OH	hydroxyl group
PBS	Phosphate buffered saline
PEG	poly (ethylene glycol)
PEO	Polyethylene oxides
PGA	Poly (glycolide)
pI	isoelectric point
PLA	poly (lactic acid)

LIST OF ABBREVIATIONS (cont)

PLGA	Poly (lactide-co-glycolide)
PPO	Polyphenylene oxide
PVA	poly (vinyl-alcohol)
PVP	poly-*N*-vinyl-pyrrolidone
SDS-PAGE	sodium doldecyl sulfate polyacrylamide gel electrophoresis
SF	silk fibroin
SEM	Scanning Electron Microscopy
V	voltage
W_d	the dried gel weight after the swelling
W_i	weight of sample before extractions
W_g	weight of sample after extractions
W_s	the weight of the swollen gels
Wt	weight

CHAPTER I
INTRODUCTION

1.1 Background

Skin is the largest organ in the body, a highly dynamic network of cells, nerves, and blood vessels. Skin does many things including: (A) protection us from the cold, heat, microorganisms, (B) preservation of fluid balance, (C) sensation from outside surrounding, (D) controlling the body temperature, (E) helping prevent a disease [1-4].

Whenever skin is damaged due to severe injury or burned by chemical, electrical, fire and etc, several functions of skin may be destroyed. Bacteria and other microorganisms are easy to access to a wound. Moreover, the vital fluids can lead to the acute, shock, illness or make a permanent scare. Wound healing can be treated by removed the burned skin surface and quickly covered by the underlying tissue or skin grafting. Skin grafting is surgical procedure in which donor site (unburned site of skin) is harvested to be replaced over a damaged wound. There are three types of skin grafting. Autologous skin grafting is the harvested skin (or graft) from a part of the body (donor site) in the same patient. Allograft is the skin grafting that obtained from the same genetically species donor as the recipient, sometimes even a cadaver [1-4]. Xenograft is the donor site and recipient is the different species as such animal to human. Allograft is can be obtain donor site from healthy subject but it is quickly rejected by a person's immune system [2-3]. The donor graft is usually smaller than the wound being repaired. Skin slits from limited donor sites in a patient with a large burn impose an even greater risk of scarring and altered pigmentation with greater apparent deformity and functional compromise in consequence,

Recently, engineering principles of biomaterials science and tissue engineering are applied to design a temporarily or permanently substitution of skin loss. Ideal therapy for wound healing must be rapidly healing with minimum scar formation and it mimics some the physiology and the mechanics of normal skin [2-3].

Promising method is an artificial skin substitute which is being researched. Artificial skin substitute is used as a three dimensional bilayer Extra Cellular Matrix comprising a highly porous graft copolymer which degrades at a specific rate in the wound and regenerates the epidermis and dermis in wound in model or patients. A bilayer structure is imitated a natural skin, wherein the upper layer functions as a temporary epidermis that is permeable a moisture. A lower layer acts as fibroblast skin regeneration layer [2].

Unfortunately, this ideal skin substitution has not yet achieved and aid wound repair not completely understood including biomaterial [3]. A commercial skin substitute's product for wound healing on market show many different natural and synthetic biodegradable biomaterials. They are used an extra cellular matrices in regenerate a skin tissue for example polypeptide, collagen, PVA, Chitosen, Atginate, Glycosaminoglycans (GAG) to create a dermal scaffold and also different healing process [2-4]. They are many advantages and disadvantages of each biomaterials such as using collagen and chitosan with Glutaradehyde (GA) as a crosslinker, less GA should be used with chitosan because its cytotoxicity [5]. Chitin and chitosan have potential for skin substitution but they have low mechanical strength that limits its use, collagen-chitosan sponge has been shown to encourage nerve growth [5]. Silk fibroin is natural protein polymer, it has physical and chemical properties can be adjust to use in biomaterial. The silk fibroin is a most protein that brought to be used in biomedicine. Several methodologies were used by the scientists to render the silk fibroin useful in biomaterial. Silk fibroin can be various forms such as suture, film, fiber, sponge and hydrogel. They are shown to support cell adhesion, proliferation, differentiation *in vitro* and also tissue repair *in vivo* [4]. It is a great challenge arises when functionalizing of a dermal scaffold has a shape, porous complex and internal architecture that promotes directly cell growth, so it is dependent on polymer adjustment [4]. Thus, the biomaterials should have biological properties such as biodegradation and biocompatibility.

The architecture of dermal scaffold is similar to a real skin, hydrogel is the best for artificial skin substitutes to choose in this study. The physical attribute shows a semisolid-liquid content or gelation condition. The properties of hydrogel depend on adding a polymer into aqueous solution. A polymer solution can be induced by

aggregation of molecules chain or with crosslinking mechanism [4]. In previous study, researcher shows varieties polymers that were tested of hydrogel property. They found their hydrogel can improves and alters a property by adjusting copolymer. Specific property of hydrogel shows softness, water absorption, hydrophilic or water soluble, non-toxic, biocompatibility [1-4]. It can be used as 3D ECM of skin substitution.

1.2 Objective

To study the silk-PVA hydrogel preparation including its physical properties by using gamma irradiation.

To evaluate biological properties of prepared silk-PVA hydrogel for artificial skin substitute.

1.3 Scope of study

A study of this research can be divided into 2 parts, to study a porous silk-PVA hydrogel preparation involving optimal composite materials and gamma ray intensity that can act as artificial skin scaffold by including 3 scopes.

1. Effect of polyvinyl alcohol (PVA) concentration addition on silk fibroin solution to gelation properties.

2. Effect of gamma irradiation to silk fibroin and silk fibroin blending PVA gelation properties.

3. Effect of salt addition on porous properties of the hydrogel.

Biological evaluation is tested to encourage a biocompatibility of porous silk-PVA hydrogel that be used as skin dermal scaffold by 2 analyses.

1. *In vitro* degradation of irradiated silk fibroin and silk fibroin-PVA hydrogel by proteolytic enzyme.

2. Cytotoxicity evaluation of fibroblast on to prepared hydrogel.

1.4 Expected results

The hydrogel prepared in this study will be a good property for cell growth and differentiation involving to skin substitutes.

Silk-PVA hydrogel can be used as an artificial skin substitutes for skin loss very well in a future.

Encouragement a silk from silk wastes as biomaterial for increment of value that are widely used in the Thailand.

CHAPTER II
LITERATURE REVIEW

2.1 Skin

The skin is largest complex organ of the body in vertebrates. It is the first part of our immune system and helps to protect our body from harmful substances. The skin helps in regulating our body's temperature, like when having a fever or physically working hard. Sweat and sebum are produced from sweat and oil glands in the skin pores. The balancing between sebum and sweat keep skin elastic and healthy. It also has third layers, first layer called epidermis, second layer called dermis and finally third layer called hypodermis [4, 6].

2.1.1 Structure and function of skin

Skin composes of three a complex nerve and blood supply to create vitamin D and other nutrients for body. The epidermis and dermis have a combined thickness of about 1-2 mm [4, 9]. The epidermis consists of a layer of keratinocytes attached to the underlying of capillary dermis through a basement membrane [4]. This section of the skin is the outermost part and contains no arteries, veins or capillaries as shown in figure 2.1. The epidermis can be divided into three distinct layers, with the basal layer being the bottommost layer of this thin epidermis. The basal layer not only contains basal cells, but also hosts the class of cells referred to as melanocytes which stimulated by sunlight to produce the melanin in the skin. This melanin is not only giving different skin complex but also protect body from the sun rays.

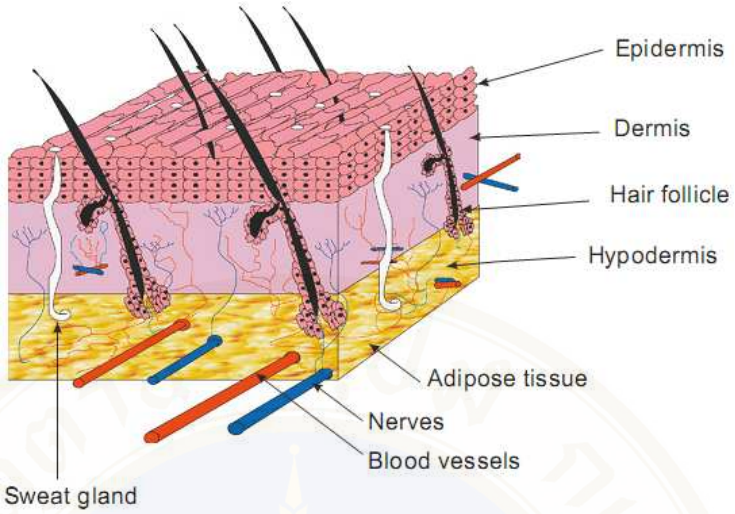


Figure 2.1 schematic structure of skin [4]

The dermis largely consists of a defined network of collagen and glycosaminoglycans which served as the skin support [4]. The rich blood supply contained in the dermis provides nourishment by diffusion to the overlying nonvascularized epidermis [8]. This part of the skin is in the middle between hypodermis and epidermis which contains fibroblast cells and sweat glands, hair follicles, sensitive nerve endings and sebaceous glands (oil glands) [9]. This fibroblast cell is not only served as the network of the skin but also serve as re-modelling enzymes producer such as proteases and collageneases, which play an important role in the wound healing process [4].

The third layer of hypodermis is mainly composed of fat or adipose tissue and a layer of loose connective tissue. The thickness of this layer varies from person to person and also from one body area to the next for example the hypodermis around the spine and nose, are much lesser than that in the area, where curves are formed. Typically, the hypodermis in women is thicker than in men, which helps to form the rounded curves in women. This layer acts as insulation and protects the internal organs from temperature variations and also acts as an energy reserve from which the body can draw as required.

Both dermis and epidermis are essential to provide the basic functions of the skin: thermoregulation, microbial defense (both mechanical barrier and immune defense), desiccation barrier, mechanical defense and wound repair, and cosmetic

appearance, pigmentation, and control of contraction. Any skin replacement must fulfill at least these five functions. In addition, it must be durable and elastic in order to provide normal function and cosmetic appearance [1].

2.1.2 Skin loss

Skin injury can occurs by accident, trauma, electrical, burning, infection etc. these injuries may be leading to permanent scare, illness and finally death depending on the depth of the injury. The depth of a burn determines and is inversely related to the skin's capacity to replace itself [1]. The degree of burn injury can be classified in three degree as shown in figure 2.2.

First degree (superficial) burns affect the outer-layer of skin or epidermis layer involving minimal tissue damage of skin surface. This kind of burn usually causes pain, red, dry and swelling. Long-term tissue damage is rare and usually consists of an increase or decrease in the skin color. A good example in this case is sunburn, which following symptoms redness, dry skin, skin that is painful to touch where pain usually lasts 48 to 72 hours then subsides and peeled [10].

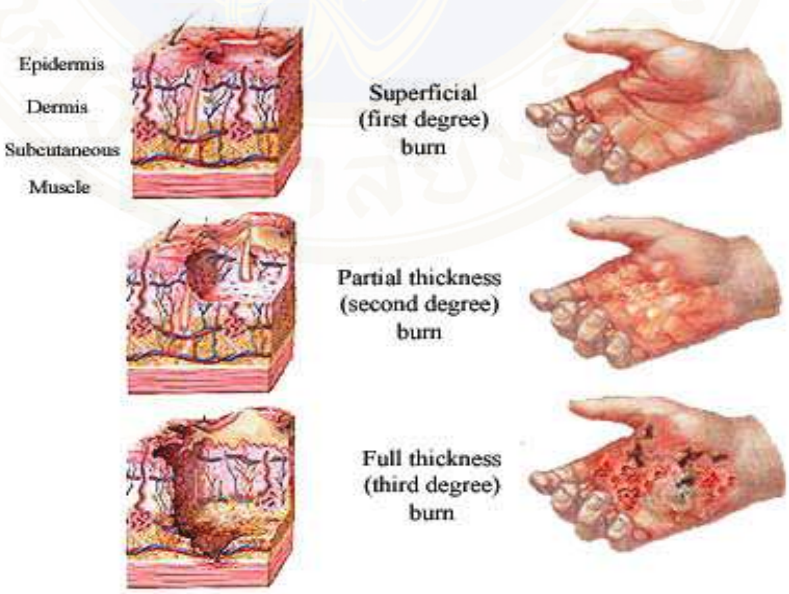


Figure 2.2 The degree of skin loss [11]

Second degree or partial thickness burns, these burns damage including epidermis and dermis layers including of sweat glands, hair follicles and blood supply.

A second degree burn is also divided into 3 sub-degree burns: Superficial second degree (partial thickness burn) mid-partial thickness burn, and deep partial thickness burn. The superficial second degree burns are defined as those burns in which the entire epidermis and variable portions of the dermis layer are destroyed. [10].

Third degree or full thickness burns, these burns include three main layers epidermis, dermis and hypodermis [1]. A characteristic initial appearance of the vascular burn tissue is a waxy white color. If the burn produces char or extends into the fat as with prolonged contact with a flame source, a leathery brown or black appearance can be seen along with surface coagulation veins. Direct exposure with a flame is the usual cause of a third degree burn. However, contact with hot liquids such as hot grease, tar or caustic chemicals will also produce a full thickness burn. The burn wound is also painless and has a coarse non-pliable texture to touch. A major difficulty is distinguishing a deep dermal from a full thickness (third degree) burn that extends just through the dermis.

2.1.3 Skin grafting

Skin loss and the healing process involve with the cell or tissue recovery on wound site. However, the healing process could take longest time depend on the remaining cell on the site. Therefore, the skin graft is typically use and promote the cell discovery on the site to the natural self ealing process. Skin grafting is sometimes done as part of elective plastic surgery procedures, but its most extensive use is in the treatment of burns especially for the third degree burns [11-13]. Recently, the healing of the skin loss can be treated by repetitive split-thickness skin grafting from unburned donor sites. These grafts are usually about 0.3-0.5 mm.thick and include the epidermal layer and thin portion of the underlying dermis. The graft is placed on the freshly excised wounds and survives by simple diffusion nutrients for first 72 hour until neovascularization of the graft occur.

2.1.3.1Types of skin grafts

The tissue graft is commonly refered to xentograft, allograft or an autograft. The allograft is a skin graft obtained from different human being called as a donor. While the xenograft is obtained from non human species (animals). Typically, both xenograft and allograft are temporary used for covering due to the rejection by human immune system. Typically, the rejection will occur within seven days. Therefore, only autograft is used for skin treatment. The autograft is a skin graft obtained from another area of the patient's own body if there is enough undamaged skin available. Skin grafting can be divided into 4 types.

Split-thickness graft is used in the case where the wound is not deep this involves the epidermis and a little of the underlying dermis. The graft is usually taken from an area that is ordinarily hidden by clothes, such as the buttock or inner thigh, and spread on the bare area to be covered. A sterile non-adherent dressing is then applied to the raw donor area for approximately three to five days to protect it from infection. While the donor site will heals within several days as well [12-13].

Full-thickness graft, these grafts involve both layers of the skin. Full-thickness autografts are more complicated than partial-thickness grafts. A flap of skin with underlying muscle and blood supply is transplanted to the area to be grafted. This procedure is used when tissue loss is extensive, such as after open fractures of the lower leg, with significant skin loss and underlying infection. The back and the abdomen are common donor sites for full-thickness grafts. The main disadvantage of full-thickness skin grafts is that the wound at the donor site is larger and requires more careful management. Often, a split-thickness graft must be used to cover the donor site to improve the healing [12-13]

Pinch grafts, quarter inch pieces of skin are placed on the donor site. These small pieces of skin will then grow to cover injured sites. These will grow even in areas of poor blood supply and resist infection [13].

Pedicle grafts with a pedicle graft a portion of the skin used from the donor site will remain attached to the donor area and the remainder is attached to the recipient site. The blood supply remains intact at the donor location and is not cut loose until the new blood supply has completely developed. This procedure is more likely to be used for hands, face or neck areas of the body [13].

Furthermore, skin donor sites are exquisitely painful, multiple donor excision procedures impose an additional large and prolonged burden of pain. Finally, to cover patients having limited donor sites with autograft alone is to cover them with epithelium but very little dermis [1].

Various risk of skin grafting may occur during the surgery such as the transmission of an infectious disease from the donor. Moreover, some grafting is unsuccessful or do not heal well which in this case it may require repeat grafting. Even though the skin graft must be protected from trauma or significant stretching for two to three weeks following split-thickness skin grafting, recovery from surgery is usually rapid. A dressing may be necessary for one to two weeks, depending on the location of the graft [10]. Schulz .J.T III at el [1] disused about many advantage of skin grafting that a split-thickness skin grafting is the standard against which all other skin-closure techniques must be judged, it suffers from several disadvantages:

- The donor site is a new wound, which is painful for the patient and, for patients with large burns, simply adds to the quantity of open wound driving their systemic inflammatory response.
- The donor site is subject to scarring and pigmentation changes.
- The dermis taken from the donor site is not replaced, leaving the donor with a permanently thinner dermis.
- The donor site is a potential site for microbial entry.
- The donor site cannot provide an unlimited supply of dermis.
- The limited supply of donor sites on a patient with an extensive burn makes it impossible to close the patient entirely with autograft.

2.1.4 Artificial skin substitutes

The skin regeneration is attractive to solve skin loss problems continuously for many decades especially to the wounds that having a several flaw. Typically, the term regeneration is usually classified by two processes [4]:

(1) Epimophic regeneration where replacement cells arise from undifferentiated cells that form a blastema from which the structures derive.

(2) Morphallactic regeneration where new cells are derived from existing tissues by cell differentiation and/or migration.

The regenerative skin or artificial skin substitute is biosynthesized material closer to covered with addressing and with skin-like properties. Artificial skin substitutes should be either permanent or temporary; epidermal, dermal or composite; and biologic or allogenic (synthetic)

1) Temporary skin substitute has designed to be placed on a fresh wound (partial thickness), it is then remove after healed.

2) Semi-permanent skin substitute is applied on the wound and remain attached to the excised wound, until eventually replaced by autogeneous skin grafts of patients.

3) Permanent skin substitute typically incorporate an epidermal and like, dermal like, or both sections as a permanent replacement

However, there are some concerns about wound healing that may affected to the design of skin substitute as mentioned by Schulz. J. T. III et. al., [1] as follows:

1. The thicker the dermal layer of a split-thickness skin graft, the less the graft contracts. Resulting that the full-thickness skin grafts contract minimally

2. Full-thickness dermal injuries heal by contraction and hypertrophic scarring, producing subepithelial scar tissue that change the morphology of dermis.

3. Partial-thickness wounds with superficial dermal loss heal with less hypertro- phic scarring.

4. The length of wound in burn cases is essentially restricted to the length of time the burn wound is open.

Therefore, the design of skin substitute for treating surgical skin loss must focus on these following properties; adherence, water vapor transportation, elasticity, durability, bacterial barrier, nontoxicity and nonantigenicit, antiseptic, hemostatic, material handling and inexpensiveness[1,14].

2.1.5 Biomaterials for skin substitutes

Biomaterials used in tissue regeneration, drug delivery, and other biomedical applications. May classified into 2 major groups synthetic and natural biomaterials. Polymers are divided into 2 classes.

2.1.5.1 Synthetic polymers

Synthetic polymers are polymers that synthesized via degree of polymerization reaction using two or three monomers. Basically, in biomedical application should be biodegradable and biocompatibility polymers.

Poly (vinyl alcohol) PVA has been used extensively for gel formation due to its cross linking ability. Moreover, it is also biocompatibility, non-toxic, hydrophilic properties. The preparation of polyvinyl alcohol can be achieved by hydrolyzing polyvinyl acetate in ethanol with potassium hydroxide. The molecular weight of PVA is between 26,300 and 30,000. The hydrolysis degree is 86.5 to 89%. It is soluble in hot water but insoluble in cold water and common organic solvents. For many applications Polyvinyl Alcohol is prepared in water solutions [5].

Polyethylene oxides (PEO), polyphenylene oxide (PPO), polylactic acid (PLA) are biomaterials used in tissue engineering, form of block or triblock copolymers. A PLA-PEO-PLA block copolymer hydrogel can be prepared simply by immersing the polymer film into organic solvent. Phase separation of PEO and PLA induces the gelation, when PEO to PLA ratio is strictly controlled and immerse into organic solvent followed by addition of water to produce a softer and more hydrophilic hydrogel [5].

Acrylamide (NIPAAm) polymer is the linear homopolymer that often formed as gel by chemical crosslinking. Generally, it become gelled when is copolymerized with another polymer having different property. Copolymers are contains a small amounts of acrylic acid and it is readily polymerized using free radical initiation in benzene and turn opaque as the temperature increase. For example, if PEO is used as copolymer, the aqueous solution is formed at low temperature and transform to relatively strong elastic gels upon heating [5].

Poly (glycolide) (PGA) is the simplest linear aliphatic polyester. It is highly crystalline material. PGA soluble in most organic solvents and exhibits high strength.

Poly (lactide-co-glycolide) PLGA is a copolymer between glycolide ans L-lactide or DL-lactide. Relationship between copolymer compositions has effected directly with mechanical and degradation property [5].

2.1.5.2 Naturally polymers

Naturally polymer has gain more interested as biomaterials due to the similarity of their structures to the human tissue. Typically, these polymers can be degraded by enzyme in body. There are two major groups of natural polymer used as biomaterials; polysaccharide and polypeptide.

Polysaccharide polymer has d-glycoside structure such as alginate, chitin, chitosan, etc.

Alginates are cell-wall constituents of brown algae (phaeophycota). They are chain-forming heteropolysaccharide or oligosaccharide made up of blocks of mannuronic acid and guluronic acid. Alginates have been used in biomedical application because of their biocompatibility, when used as biomaterials for implantation, tissue engineering scaffold, and alginate have to be crosslinked. Alginate can be easily crosslinked by calcium ions to form ionic binding between alginate molecules. Alginate can be covalently crosslinked by ethylenediamine in the presence of water –soluble carbodiimide, the carbodiimide will induce crosslinks between carboxylic acid and amine group. The crosslink by covalent bond is biodegradable readily and reduce foreign-body reactions for skin wound. Alginate can also be fabricated into fibers; they can be used to non-woven fibers. Alginate fiber dressings have been used frequently on both partial and full thickness wound [5].

Chitin and Chitosan, chitin is mostly found in nature from shells of crustaceans, insect exoskelletons, plankton. Chitin is a homopolymer of N-acetylglucosamine linked in a beta configuration forming a long chain linear polymer. It is insoluble in most solvent. Chitosan is a derivative of chitin by removing most of the acetyl groups of chitin using strong alkalis to gain a soluble product. Chitosan is a semi-crystalline polymer and the degree of crystallinety is a function of the degree of deacetylation. Chitosan is normally insoluble in both organic solvents and aqueous solutions at pH above 7, it dissolves readily in dilute organic acids solution and it can be soluble in a limited extent in dilute inorganic acids except phosphoric and sulfuric acid. The pH-dependent solubility of chitosan is a very useful property. Chitosan shows antimicrobial, antifunic activities and also does not evoke any inflammatory or allergic reaction [5].

Polypeptide is another group of natural polymer used as biomatierials.

Collagen is the most abundant protein in mammalian tissues. Collagen fibers consist of a main part of tendons and act as major part of skin and formed the matrix or cement material in human bones where bone mineral precipitate. The core function of collagen is the mechanical reinforcement of the connective tissues of vertebrates. The polypeptide chain of collagen contains 20 different amino acids. The variation in specific amino acid sequence gives rise to different types of collagen as type I, II up to XIX. Type I collagen is mostly found in skin, tendon and bone, type III in blood vessels. Crosslinking of collagen makes it become more stable and increases tensile strength and visco-elasticity especially in skin and tendon. The crosslinking can be occurred naturally within the body reaction involves lysine chains. Intramolecular crosslink are formed by an Aldol condensation reaction of two aldehyde groups. Intermolecular crosslinking is formed if the aldehyde group reacts with ε-amino acid of a hydroxyl lysine residus of an adjacent helix [5].

Gelatin is a protein produced by partial hydrolysis of collagen extracted from the bones, connective tissues, organs and some intestines of animals. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling. Its chemical composition is closely similar to that of its parent collagen. These proteins form a compound (triple) helix in aqueous solution.

Silk is one kind of protein structure comes from native silkworms or spider that consisting of polypeptide chains. Polypeptides are comprises of different twenty amino acid, which crosslinked by peptide bond. Differences of amino acid on polypeptides have a specific properties, which provides unique mechanical properties with biocompatibility and relative or environmental stability. Silks are characterized by a combination of highly repetitive primary sequence that leads to significance in secondary structure. Silk is usually called nonbiodegradable biomaterial but it can be degradation or modified by enzymatic or ionizing radiation [5]. Silks can be applied to various formation of biomedical application such as suture, biosensor, and scaffold of tissue engineering [5, 15].

2.2 Silks

Silk is a strong natural fiber, especially, chemical and temperature stability. It is long known that Thailand is a source of high quality producing silk fibre. There are two types of silk: mulberry, silk (*Bombyx mori*) and wild silk. Silkworms produce silk fiber upon one stage in their life cycle. The lifecycle of *bombyx mori* lasts 55-60 days and passes through a series of development stage or molts and cocoon formation occurs around 26 days in the cycle [15]. Thai silk is one of mulberry worms but it differing somewhat in the appearance. It is yellower in color, the filaments are coarser, and it has more silk gum than normal mulberry silk [16]. Silks have mechanical properties, in addition to environmental stability, biocompatibility, controlled proteolytic biodegradability, morphologic flexibility and the ability for amino acid side change modification to immobilize growth factors. 25-30% of silk cocoon mass is sericin which is removed in degumming process by alkaline solution [17]. The greatest importance is the sericin glue-like proteins are the major cause of adverse problems with biocompatibility and hypersensitivity to silk, silk is susceptible to proteolytic degradation in vivo and over longer time in vivo will slowly be absorbed.

Silk is defined as non-degradable accorded to US Pharmacopeia, however it can degradable spent for long time period and slowly absorbed in vivo and silk lose the majority of their strength within one year. Silk fibroin can be classified as enzymatically degradable polymers because natural polymers like collagen and silk degrade via the action of enzyme protease. The enzymatic degradation of biomaterials is two step processes. The first step is adsorption of enzyme on the surface of the substrate through surface-binding domain and the second step is hydrolysis of the ester bound. A relation between *in vitro* and *vivo* of degradation of silk fibroin in several forms such as film, fiber, and the characteristics of silk degradation behavior vary a several type of proteolytic enzymes. Silks were tested upon incubation with proteolytic enzymes, focusing on three types of enzymes protease was more aggressive than alpha-chymotrypsin or collagenase. The average molecular weight of silk biomaterial after degradation follows the order protease XIV < collagrnase IA < alpha-chymotrypsin. The protease XIV does not only degrade silk fibroin, but also directly degraded the fibroin sheet into peptides and amino acids [21]. The rate of silk fibroin

degradation depends upon the structure, morphology and mechanical and biological conditions at location of implantation. The weight loss was accompanied by change in average molecular weight of silk and degrades of amorphous portion of silk structure. Thus, crystallinity portion of the silk structure is increased [21]. The difference in degradation was due to increased surface roughness or difference in content or distribution of crystallinity. Therefore, silk should be changing crystallinity, pore size, porosity and molecular weight distribution (MWD) of silk fibroin. These decreased in MWD may disrupt ordered structures and reduce cross-links resulting in faster degradation [17-21].

Figure 2.3 *Bombyx mori* cocoons [22]

2.2.1 Silk structure

Fibroin is water insoluble protein silk fibers and sericin is the water-soluble protein that binds the fibroin fibers together [23] as shown in figure 2.4. Silk fibroin is fibrous protein polymers consist of repetitive protein sequences. It exhibits impressive mechanical properties as well as biocompatibility, biodegradation, bioresorbability, haemostatic properties, non-toxicity, low-antigenicity and non-inflammatory response [23-24].

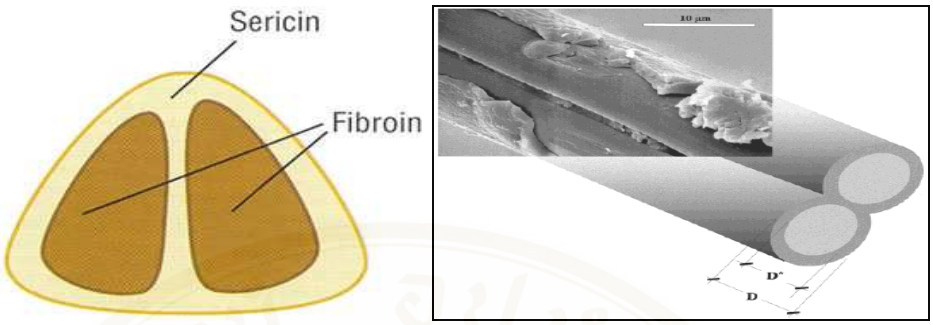


Figure 2.4 Structure of silk fiber [25]

The silk fibroin molecule consists of heavy and light chain polypeptides of ~350 kDa and ~25 kDa, respectively, connected by a disulfide link. The fibroin protein is composed various amino acids i.e., glycine (43%), alanine (30%), and serine (12%), which form antiparallel beta-sheets spun fibers. The spun fibers molecules comprise a crystalline portion more than of thirds and an amorphous region one-third. This crystalline portion is amino acid sequence in form of antiparallel beta-sheet which resulting in fibers stability [23]. These structures are permitted tight packing of stacked sheets of hydrogen bonded anti-parallel chains of the protein. Large hydrophobic domains interspaced with smaller hydrophilic domains foster the assembly of silk and the strength and resiliency of silk fibers [17, 20, 26].

The rearrangement of silk secondary structure is compost of random-coil or unordered structure (silk I), beta-sheet structures (silk II) and an air/water assembled interfacial silk (silk III) with a helical structure (unstable structure) as shown in figure 2.5. The silk I structure in protein arrangement is the water-soluble state it can convert to silk II structure upon exposure to heat or physical treatment methanol or potassium chloride. Hydrogen bonding is important in fibroin because it inter-crosslinked in crystalline domains of fibroin. Strong hydrogen bonds and van der Waals forces generate a structure that is thermodynamically stable [15-19].

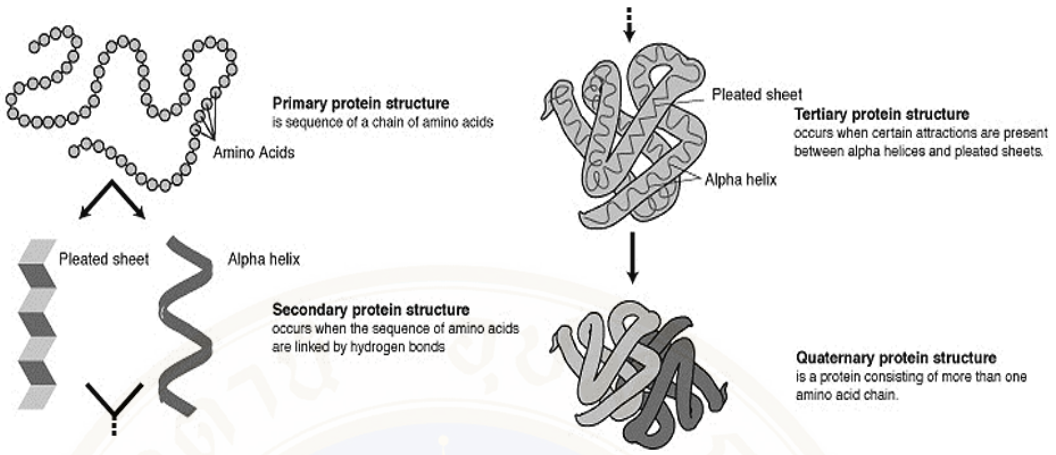


Figure 2.5 Silk structure is a protein formation consisting of 4 main structures [27]

Silks as biomaterial application are used in several different formation can be form from aqueous or solvent formulation. It must be dissolved in aqueous solution, followed by desired processing formats. Tissue engineering can shows an application of silks that can applied to healing, regenerate and repair very well. Past decade of silk research shown a good biocompatibility and match with application such as silk apply in sponge scaffold in bone tissue or wound dressing use film, hydrogel. Thus, selection of silk formation is important, especially skin loss healing. The hydrogel formation can show physical attribute similar to real skin, it can easily fabricate into aqueous solution to grab into water with silk molecules. Their properties depend on property of silk to modify a hydrogel. Hydrogel combining with property of silk can promote cell growth and differentiation.

2.3 Hydrogel

The hydrogel is a three dimensional structures crosslinked each other. Hydrogels are composed of hydrophilic homopolymer or copolymer networks and can swell in the presence of water or physiological fluids, chemical crosslinks (covalent bonds) or physical junction e.g. secondary forces, crystallite formation, chain entanglements [28]. Typically, the hydrogel can be formed with the polymers containing carboxylic acid group Water molecules are attracted to the negative charges by hydrogen bonding in the gel matrix forming the crosslinked structure between random-coil structures. When gelation occurs, the main structure of hydrogel is beta-sheet (secondary structure), it is recognized. It arise from a combination of inter-intramolecular interaction and hydrophobic, hydrogen bonds interaction leading to beta-sheet formation. All of these interactions are reversible and it can be disrupted by changes in physical conditions [38].

Figure 2.6 Example of hydrogel conformations [29]

More specifically, in situ forming hydrogels can provide a means for simple, custom-made therapeutics and diagnostics. A polymer solution can be prepared and allowed to gel in situ, after photopolymerization, chemical crosslinking, ionic crosslinking, [33-35] or in response to an environmental stimulus such as temperature, pH or ionic strength of the surrounding medium [36]. Their sensitivity to the thermal environment is useful as temperature to stimulate for their gelation when temperature is increased or decreased from ambient to physiological.

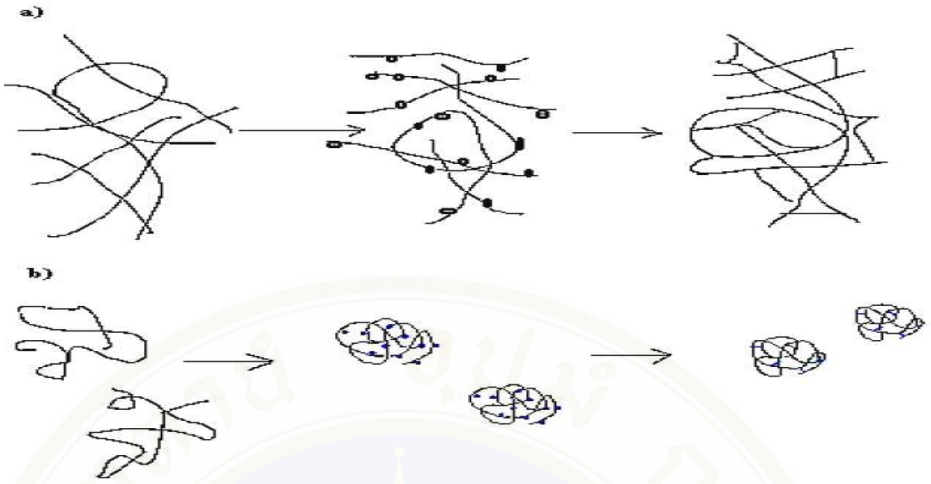


Figure 2.7 Schematic representation of (a) intermolecular crosslinking due to the fixation of entanglements in concentrated polymer solution and (b) intramolecular crosslinking in diluted solution of isolated chains. Dots on macromolecule before reaction denote radical sites [32].

2.3.1 Factor effect on sol-gel transition

Gelation phenomenon is a sorting of molecules in the environment with physical and chemical condition. It can binds together as 3-dimensional structure (Matrix). When the molecules of polymer separating and relaxing. Their will combine and bind slowly to become the 3-dimensional structure of proteins. The viscosity will increase rapidly. In the final phase of the gel that will have properties of solids flexible. This process can be done by the heat, concentration, or by other factors such as the power to adjust

2.3.1.1 Temperature and concentration

Heat will play an important role to determine the proportion of polymer and relaxing the polymer linked to a network. The temperature was selected for study as an artificial *in vitro* control of the process on the basis of potential impact on chain-chain interactions. Akira Matsumoto et al, [38] shows the result of temperature effecting on gelation time, 37 degree Celsius the gelling time are fast than room temperature.

Protein has a lower concentration than that used in the structure and then cannot make a gel. Since the protein quantity is not enough to cause a sort of a shared infrastructure. Increment of protein concentration is result in a networking structure and the concentration of protein that will make the appropriate gel. Increasing concentration of protein is the strength and texture characteristics of the touch hardened gel. In silk hydrogel, the occurrence of sol-gel silk fibroin depended on the concentration of the protein (Silk fibroin solutions

at concentrations less equal than 5 wt % have been previously studied with respect to hydrogel formation), Random coil to beta-sheet structural transitions were noted during the process of gelation [37].

2.3.1.2 pH-values

pH level is affected the charge of the protein. This will affect dissolution. It also affects the breakdown and relaxing the condition of the protein structure. Therefore, to maintain the appropriate level of pH will help the gel structure is unchanged by the balance between molecules. They also make adjustments to relax the rate of protein during the order and rate of protein-protein a balanced. Jingxin Zhu et al, [39] showed that pH was induced to formed gelation as quickly at lower pH, it also suggested that the optimal pH value for gel formation was fairly closed to the isoelectric point of hydrogen-chain bound. Jingxin Zhu et al, [39] discussed that lowering the pH suppressed repulsive forces between the negative charges in two regions of the fibroin molecule. Thus the hydrophobic blocks approached one another to produce hydrophobic interactions and induced the gel formation.

2.3.1.3 Value Ionic strength

Ionic strength in protein solution can change protein solubility by changing the surface tension of protein, however at a higher ionic strength of protein solution the solubility of protein is decreased. Kelven Pagel et al. [40] explained that metal ion can binded with side groups of amino acid as a histidine due to it high electron donor characteristics to transformed from alpha-helices to beta-sheet structure, therefore it is also affected to the gel formation as well. The gelation rate of regenerated silk fibroin aqueous solutions was studied with varying above these factors. Rheological evaluation of dilute solutions of silk fibroin from *B. mori* revealed that the protein chains tend to form clusters by ionic interaction between COO^- ions of amino acid side chains in the fibroin and divalent ions such as Ca^{2+} or Mg^{2+}. Through these interactions, the pH of silk fibroin solutions with Ca^{2+} ions was significantly lower than that of silk fibroin solutions in the absence of these ions, resulting in stronger potential for the formation of a beta-sheet structure through hydrophobic interaction [37].

2.3.1.4 Gamma irradiation

A gamma ray is a packet of electromagnetic energy (photon) emitted by the nucleus of some radionuclides following radioactive decay as shown in figure 2.8. The three radionuclides by far most useful are cobalt-60, cesium-137, technetium-99m and americium-241. Gamma photons are the most energetic photons in the electromagnetic spectrum. Gamma rays are a form of electromagnetic radiation (EMR) Electromagnetic radiation can be described in terms of a stream of photons, which are massless particles each traveling in a wave-like pattern and moving at the speed of light. Each photon contains a certain amount (or bundle) of energy, and all electromagnetic radiation consists of these photons. Gamma-ray photons have the highest energy in the EMR spectrum and their waves have the shortest wavelength. Gamma-ray photons generally have energies greater than 100 keV. The high energy of gamma rays enables them to pass through many kinds of materials, including human tissue. Very dense materials, such as lead, are commonly used as shielding to slow or stop gamma photons [41-42] as shown in figure 2.9.

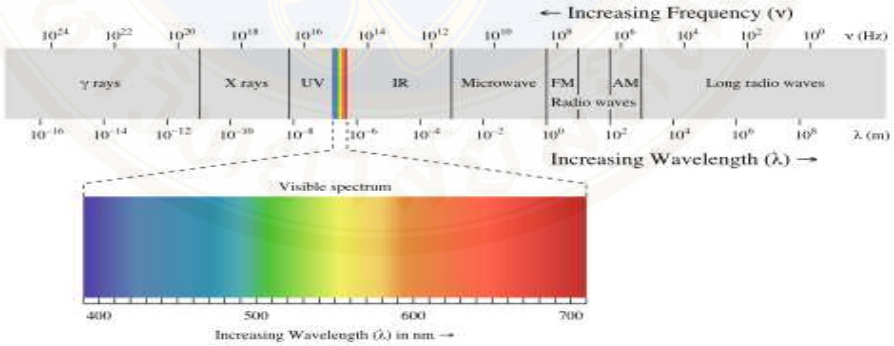


Figure 2.8 Electromagnetic spectrum [42]

Many papers show a result of gamma ray intensity on gelation property by the researcher trying to modify or adjust a hydrogel property by physical and chemical condition to improve attribute and property of hydrogel that can enhance a wound healing. According to Amornthep kojthung et al.[7], found that silk fibroin is radiated from gamma radiation, it can more dissolved and degraded than non radiated silk fibroin and can be induced to attach and proliferate faster than non radiated silk

fibroin. Gamma radiation will deform or damage the arrangement of a secondary protein structure. Therefore, the analyze study about any properties of gamma-radiated silk fibroin are very importance to development medical biomaterial. Su Jung You et al. [44] shown that ^{60}Cobalt gamma-ray Irradiative cross-linking techniques can induce chemical reactions to modify biopolymers as PVA/gelatin hydrogel at low temperature and radiation doses shows effected on hydrogel properties by increasing gel content, gel strength but decreasing swelling, elongation. Nho Y.C. et al., [48] reported that organic solvent as PVA/PVP with kappa-carrageenan, silk powder composition and irradiation dose had a greater influence on swelling, tensile strength and gel content. Intensity of irradiation can show a physical characteristic of hydrogel at high dose (25 KGy) of PVP hydrogel exhibits transparent solid gel and low dose (5 KGy) exhibits transparent fluid gel, when added a PEG, PEO can increase in the crosslinking density and elasticity of the PVP gel [49]. Accordingly, it has a many means yet to improve and modify a hydrogel as use an acetalization before irradiation to give a material better mechanical, physical property [50]. In irradiated PEG or PVA, the crosslinks consist of C–C bonds, this gel will not be biodegradable [51].

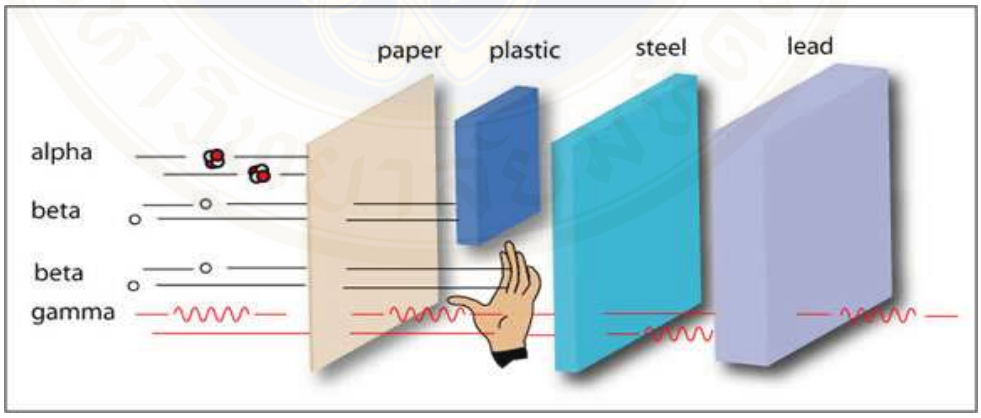


Figure 2.9 The penetration of gamma-rays [41]

2.3.1.4.1 Formation of macroradicals

Formation of Macroradicals of polymer solution is subjected to ionizing radiation; reactive intermediates are formed in the macromolecules. This can result from the direct action of radiation on polymeric chains and from the indirect effect, i.e. reaction of the intermediates generated in water with macromolecules. The

three main reactive species formed in water upon irradiation hydrated electrons, hydroxyl radicals and hydrogen atoms electrons exhibit low reactivity towards neutral, hydrophilic hydrogel forming polymers [43]. Hydroxyl radicals have been shown to be the main species responsible for reactivity transfer from water to the polymer chains. They bind hydrogen atoms from macromolecules,

2.3.1.4.2 Transformation of macroradicals

The most important reaction of macroradicals is Transformation of macroradicals. Intermolecular crosslinking are recombination of radicals localized on two different macromolecules. The many of the initially formed macroradicals undergo side reactions. These side reactions include reactions between two radicals, as intramolecular crosslinking as well as inter and intramolecular disproportionation, and then also processes involving one radical, as hydrogen transfer or chain scission. Usually the possibility to control recombination and disproportionation is very limited because radical structure. The probability that two recombining radicals are localized on different chains is relatively high when polymer chains inter-penetrate and some physical entanglements may become fixed when the entangled chains join to the network in at least two points encompassing the entanglement site. Nevertheless, some influence on the crosslinking yield and network structure can be expected by changing the initial proportions between various radical structures that may be more or less prone to recombination, may lead to various gel microstructures [34]. Additives can alter the radiation-induced processes in aqueous solutions of polymers. Crosslinking agents and monomers are applied in order to increase the yield of crosslinking, modify the gel structure or initiate grafting reactions.

Hydrolgel as an artificial skin substitute

hydrogel need to degrade at a rate commensurate with new tissue formation to allow cells to deposit new extracellular matrix (ECM) and regenerate functional tissue. Ideally a skin scaffold should possess the following characteristics to bring about the desired biologic response: (1) three-dimensional and highly porous with an interconnected pore network for cell/tissue growth and flow transport of nutrients and metabolic waste, (2) biodegradable or bioresorbable with a

controllable degradation and resorption rate to match cell/tissue growth *in vitro* / *in vivo*, (3) suitable surface chemistry for cell attachment, proliferation and differentiation, (4) mechanical properties to match those of tissues at the site of implantation, and (5) be easily processed to form a variety of shapes and sizes [30]. Scaffold characteristics such as interconnectivity, pore size/curvature, microporosity, macroporosity and surface roughness influence cellular responses, but they also collectively control the degree of nutrient delivery, penetration depth of cells and metabolic waste removal. It is also important to allow cell-seeded scaffolds to be subjected to a strain environment. The pore size employed may also be dependent on the tissue-type desired. The scaffolds with pore sizes wide range 38-150μm has been successfully used for regeneration of skin in burn patients [30-31].

CHAPTER III
MATERIALS AND METHODS

The methodology in this study can be divided into 2 categories that are preparation study of porous silk-PVA hydrogel under gamma irradiation and physical properties is tested for support a physical artificial skin substitute property. Biological evaluation study indicated this porous silk-PVA hydrogel is able to be used as artificial skin substitute.

3.1 Materials

3.1.1 Silk cocoons of *Bombyx mori var. Nangnoi Sisaket-1*, (obtained from TINT Thailand)

3.1.2 Mouse Fibroblast cells (3T3 ATTc USA)

3.1.3 Calcium chloride dehydrate, $CaCl_2.H_2O$ (MERCK, Germany)

3.1.4 Salt particle (NaCl) (analytical reagent Ajex Finechem)

3.1.5 Poly (vinyl-alcohol) (mol wt 30,000-70,000 R&D grade Sigma, USA)

3.1.6 Ethanol C_2H_5OH (MERCK, Germany)

3.1.7 Phosphate buffered saline (PBS), pH 7.4

3.1.8 Cell culture media (5% fetal calf serum in RPMI media)

3.1.9 Polystrene dish cell culture 60 mm×15mm style (Corning USA)

3.1.10 Protease type XIV from *Streptomyces griseus* (Sigma, USA)

3.1.11 Regenerated cellulose tubular membrane (Cellusep T4, Nominal MWCO; 12,000-14,000 USA)

3.2 Equipments

3.2.1 Gamma irradiator (Gammacell 220 excel, MDS Nordian)

3.2.2 Fourier transforms infrared spectroscopy (FT-IR) (Spectrum GX, Perkin–Elmer)

3.2.3 UV-VIS spectrophotometer (Lambda 35, Perkin Elmer)

3.2.4 Inverted light microscope (CKK 41, Olympus)

3.2.5 SEM (XL30&EDAX, Philips)

3.3 Methodology

3.3.1 Silk solution preparation

Silk solution is prepared by silk cocoons of *Bombyx mori* are cutted a pieces and then the removal of the sericin by the degumming method.

The silk cocoons are degummed in 0.5 % w/v Na_2CO_3 solution at ratio silk 1: solution 50 and boiled with hot water 30-45 min and then washing with water to discharging Na_2CO_3 .Dried at 60°C for 3 hour.

A 0.5 degummed silk is dissolved in 10 ml of $CaCl_2.H_2O$: C_2H_5OH:H_2O by mole ratio 1:2:8 solution. The silk fibroin solution is then poured in cellulose membrane, silk solution into cellulose membrane is dialyzed into distilled water for 2 days change water every 30 minute; all solutions are stored at 7°C before used.

3.3.2 The study of gamma irradiated porous silk−PVA blended preparation

3.3.2.1 Effect of polyvinyl alcohol (PVA) addition in silk fibroin solution for gelling properties.

Addition of PVA in silk solution at silk: PVA ratio 1:0.1, 1:0.3, 1:0.5, 1:0.7 and 1:1 respectively.

Optical density measurement of samples every 1 hour at 550 nm for gelation

3.3.2.2 Effect of gamma irradiation to gelation properties of silk fibroin and silk fibroin−PVA blended solution.

Preparation of silk blend PVA using optimium condition of previous study with minimal gelation time, all samples are irradiated by gamma ray at 1, 5, 10, 15, 20, 25, 30, 40, 50 KGy respectively, non-irradiation are used as a negative control.

Optical density 550 nm and FT-IR measurement for structure change, degree of swelling.

3.3.2.3 Effect of salt addition on porous properties of the hydrogel

Preparation silk-PVA blended in solution with addition of salt particles 30-150 microns.

Variation of salt particles to silk-PVA solution at weight ratio 1:0.01, 1:0.03, 1:0.05, 1:0.07 respectively.

All prepared solutions are subject to gamma irradiation at minimal intensity of gelation.

SEM measurment for the morphology of silk-PVA hydrogel structure.

3.3.3 The biological evaluation of hydrogel for artificial skin substitutes.

3.3.3.1 Cyto-toxicity evaluation of fibroblast on to prepared hydrogel

Cell cultures of silk-PVA hydrogels are prepared from Scaffold for cell culture for cell growth proliferation and differentiation Inverted light microscope is observed and monitored a cell growth, proliferation, differentiation and viability.

Skin scaffolds are immersed in proteases and PBS as negative control, *In vitro* degradation tests for skin scaffold for everyday.

3.3.3.2 *In vitro* degradation of irradiated silk fibroin and silk fibroin-PVA hydrogel by proteolytic enzyme

Silk-PVA hydrogels are prepared from previous study that is incubated in protease enzyme and PBS 7.4 as negative control.

% mass loss is measures a weight loss of hydrogel everyday.

Silk protein structure degraded is measured everyday at 280 nm by UV-VIS Spectrophotometer.

A molecular-weight (203–15 kDa) of silk protein structure is degraded to determine by Gel electrophoresis

CHAPTER IV
RESULT AND DISCUSSION

The preparation of artificial skin substitutes in this study was carried out by mixing silk fibroin solution with PVA at various mass ratios from 1:0 till 1:1. The physical changes of these samples were monitored throughout the experiment. The structural change associated with gel formation was observed by optical density change using UV spectrophotometer and protein structural rearrangement by FT-IR. The results are shown in figure 4.1

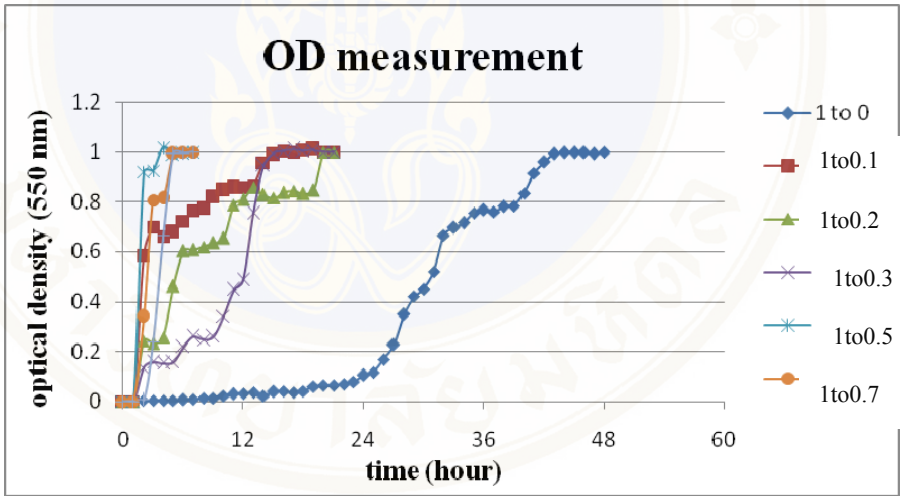


Figure 4.1 Gelation time of silk-PVA composite was measured by Optical density at 550 nm

The silk blended with PVA at varies ratio were monitored by optical density change at 550 nm, upon gelation period. The increasing of PVA content shows the faster gel formation period as shown in figure 4.1. The optical density of silk fibroin without PVA increase within 24 hour of gelling process, the optical density continue increasing to 1:0 of about 40 hour after gelling process, the duration of gel formation period. By increasing PVA content the gelatio period is shortened

significantly to about 12 hour at PVA ratio of 1:0.1 to 1:0.3. However, the silk-PVA ratio of 1:0.5 and higher has the fastest formation rate within 6 hours. These results show that the higher PVA content decreases a gelation time.

4.1 Effect of gamma irradiation to gelation properties of silk fibroin and silk fibroin−PVA blended solution

The silk–PVA composite at varies ratio were irradiated by gamma irradiation. All samples were irradiated at 5, 10, 15, 20, 30, and 40 kGy. The irradiated samples were monitored by optical density at 550 nm after irradiation. The gel formation of irradiated silk fibroin is shortened as irradiation dose increased. The silk fibroin at 5 kGy can become gel within 14 hours. Irradiated silk solution at 10-25 kGy can become gel within 10-7 hours. The shortest gel formation period of 1 hour for silk fibroin solution can be achieved with the irradiation exposure at 30 kGy. Thus, indicating that the gamma ray can activate a crosslink between silk fibroin and PVA.

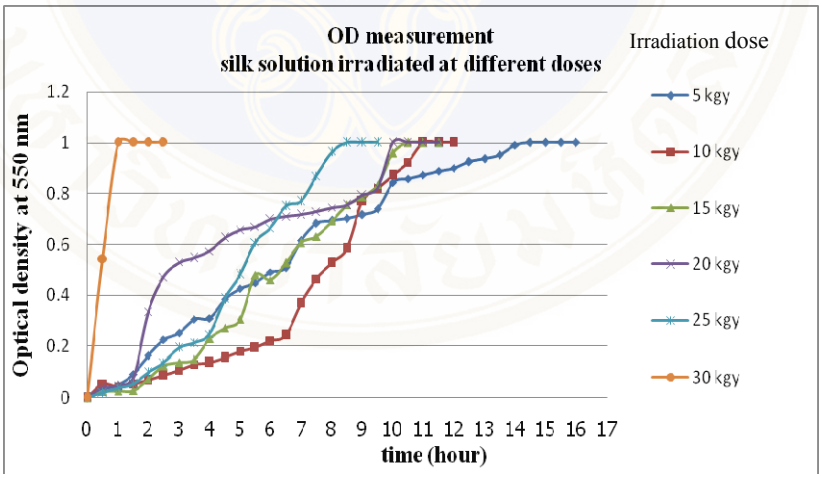
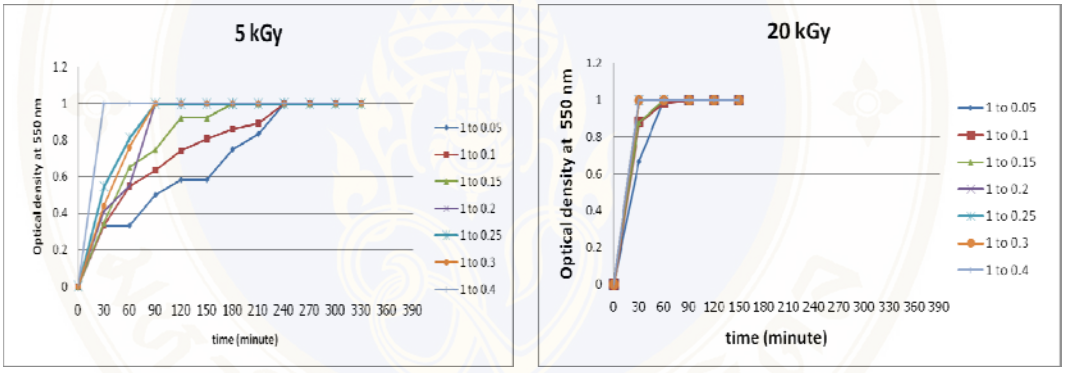


Figure 4.2 silk sol-gel transitions at different irradiation doses was measured by OD 550 nm

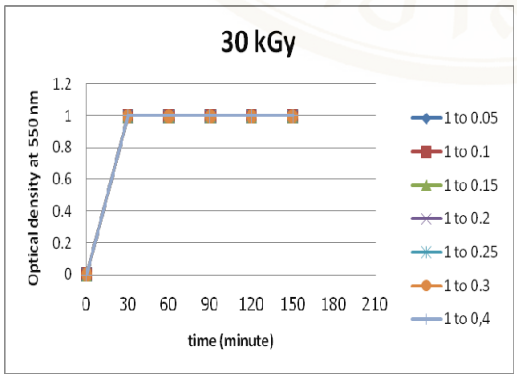
The effect of PVA content in gel formation

According to the irradiation on silk-PVA blended at each dose as seen in figure 4.3, the sample with a higher PVA content can become gel at shorter period of time. As increase the irradiation doses from 5 kGy to 20, 30, and 40 kGy in figure 4.3 (a, b, c, d) respectively. Especially, when the irradiation dose higher than 15 kGy the samples can form gel within 30 minute regardless of PVA contents. Figure 4.3 a) show that the silk-PVA at ratio of1:0.5-1:0.1 can become can become gel after 24 hours with the increasing of PVA ratio shorter, the gel formation period down to 60 minute at silk PVA ratio of 1:0.3. The increase of irradiation dose for 5 kGy to 20, 30 and 40 kGy can shorter the gelation period significantly.

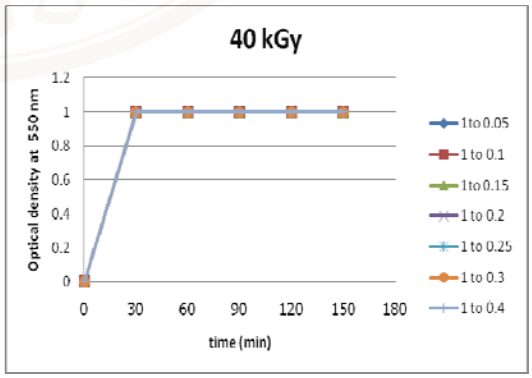


(a)

(b)



(c)



(d)

Figure 4.3 optical density silks blended PVA at varies ratio (a) 5 kGy, (b)20 kGy, (c)30 kGy, (d) 40 kGy respectively

This can be explained by the OH group in PVA structure was energized by the gamma ray during irradiation and form as microstructure. This can bind with OH linkage between .polypeptide in silk fibroin structure to form the silk-PVA network. The increase of irradiation dose and PVA content can induce the crosslinking network for these two structures, thus decrease the gelation time and increase the gel strength.

4.2 degree of swelling test

The degree swelling study of silk-PVA hydrogels irradiated at 15 to 70 kGy were performed in distilled water. The swelling test of hydrogel can indicate the water-protein network of crosslink gel by irradiation the water occupancy within hydrogel. If the cuessling degree is high, the water occupancy would be increase and the water swelling degree would also increase as well. The water absorption in all silk-PVA blended samples started at low yield at 15 kGy and gradually increases by increasing the irradiation upto 25 kGy which gives the maximum yield and then decreasing gradually as seen in figure 4.4. All of the prepared hydrogels were completely dissolved within 48 hour in distilled water. Swelling behavior of various silk-PVA samples are in the similar pattern, the high swelling degree obtained at 25 kGy. The increasing in PVA ratio in silk solution gives % higher swelling degree. Indicating that the PVA ratio and irradiation dose can induce the crosslink protein of silk fibroin, the optimum energy activates silk protein for network rearrangement to occur around 25 kGy. However, by increasing the activate energy will not increase the water absorption possibly due to the large amount of short micro structure are formed and binding eith PVA and form tight protein network hydrogel which reduce the water absorb ability.

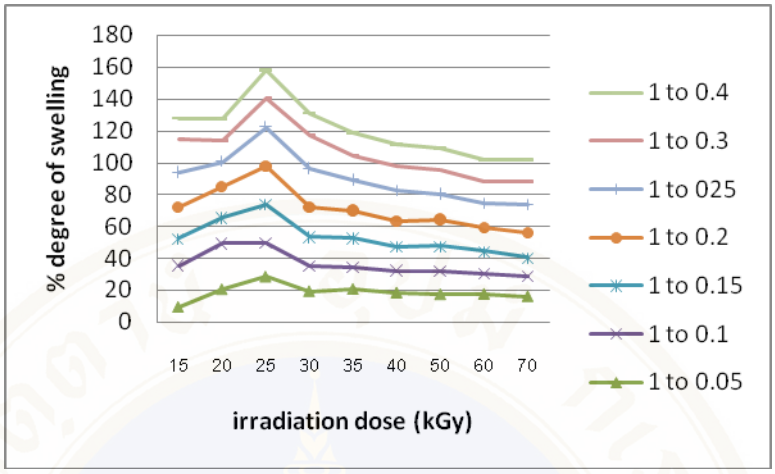
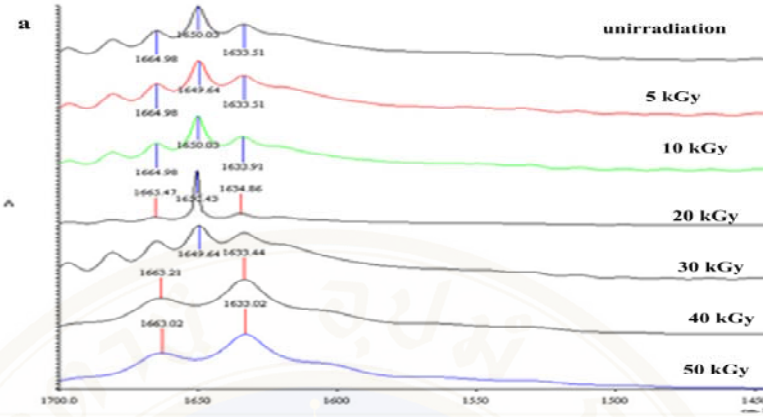


Figure 4.4 degree of swelling of silk-PVA hydrogel composite

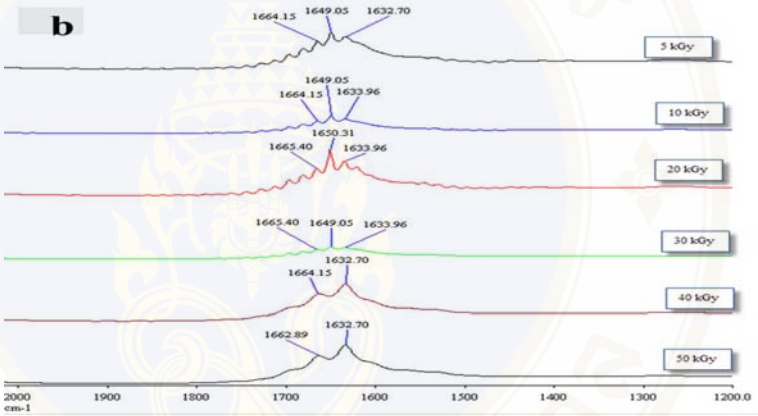
4.3 FT-IR spectrum for silk –PVA hydrogel structural change

Typically, the IR absorption of silk solution is composed of the vibrational transition bands of C=O stretching or amide I (1700-1600 cm^{-1}), N−H deformation. The analysis was carried out by comparing the absorption of IR spectrum of silk fibroin solution and that of composite silk-PVA samples at different concentration and different irradiation doses. Figure 4.5 showed the IR spectrum of various silk fibroin solutions at different irradiation doses. The native silk solution has strong absorption band of amide I. The irradiated silk fibroin show the decreasing of alpha-helice or random-coil at about 1660 cm-1 to beta-sheet at 1650 cm-1 as increasing the irradiation doses especially for the pure silk solution, indicating that the sol-gel transition can be induced by gamma radiation.

The similar change can also be observed when blended silk solution with PVA can see in Figure (4.6 b) IR absorption band of alpha-helice at1660 cm-1 to beta-sheet at 1630 cm-1 when exposed to the gamma ray. The peak area ratios of beta-sheet (1660 cm-1) to hepha-helice (1660 cm-1) of irradiated silk-PVA blended are summarized in figure 4.6. This showed that irradiated of gamma ray higher the 30 kGy can induce the structural change to beta-sheet significantly in all samples. Therefore the optimum irradiated dose to activate the gelation gamma ray is about 50 kGy regardless of PVA content.



(a)



(b)

Figure 4.5 ATR-FTIR spectra (a) silk solution, (b) silk/PVA respectively

From figure 4.6 the addition of PVA, silk can also change the beta-sheet to alpha-helice ratio in irradiated sample possibly due to the PVA can produce more OH group when it irradiated, the OH radical combines with micro-molecules or session chain of silk fibroin structure and rearrange in more order structure (as beta-sheet). Thus, the beta-sheet to alpha-helice ratio shown in figure 4.6 have increase significantly after irradiation at higher 30 kGy for all sample. This also agreed with the swelling test and OD measurement which the irradiation dose between 25-40 kGy shows the highest absorbent of the hydrogel

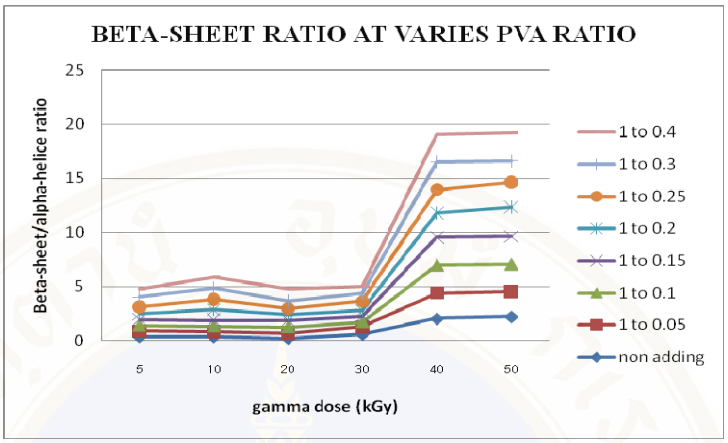


Figure 4.6 beta-sheets to random-coil ratio

4.4 *in vitro* degradation test

The *in vitro* degradation test of composite hydrogels at gamma ray 10, 30 and 50 kGy were tested by incubated in protease at 1, 0.1, 0.01 mg/ml, PBS solution as a negative control. The supernatant were collected for every samples for protein structure from the hydrogel every 5 day, the gel were also weighted for weight loss evaluation and shown in figure 4.7. The example of weight loss at degraded enzyme concentration of silk: PVA (1:0.15) showed that the irradiated silk-PVA sample at higher dose have a slow degradation of all sample possible dui to the low crosslink degree compare to the sample irradiated at network. Moreover, the weight loss after 10 day of enzyme remains constant possibly due to the loss of enzyme activity. The 1:0.05 and 1:0.3 ratios of PVA in hydrogel cannot detect because both of these ratios are consist of lower and higher of PVA causing the hydrogel is broken during enzymatic degradation test.

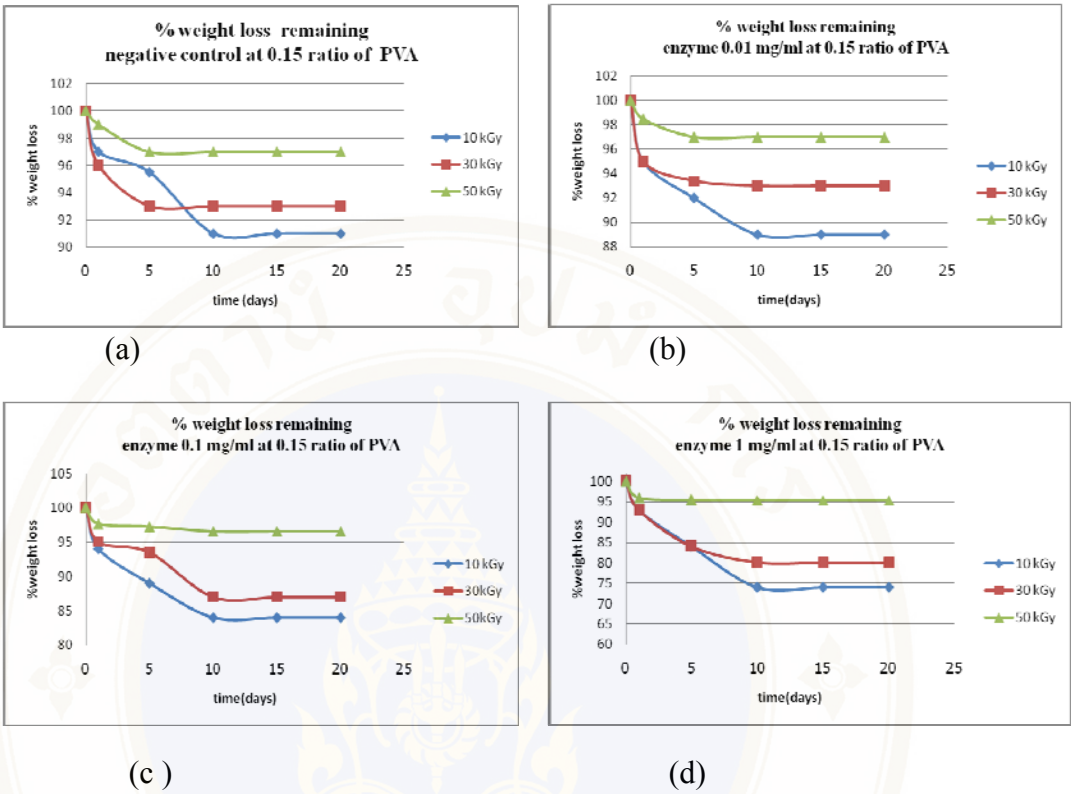
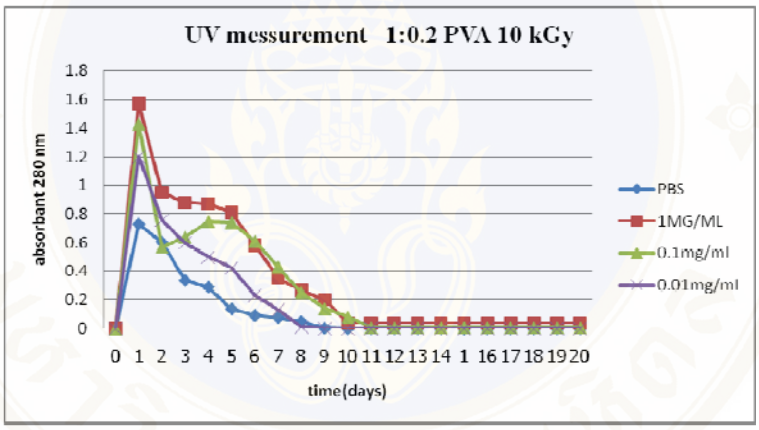


Figure4.7 %weight loss remaining of 1:0.15 PVA ratio of hydrogels were tested by (a) PBS solution (b) 0.01mg/ml, (c) 0.1mg/ml, (d) 1mg/ml

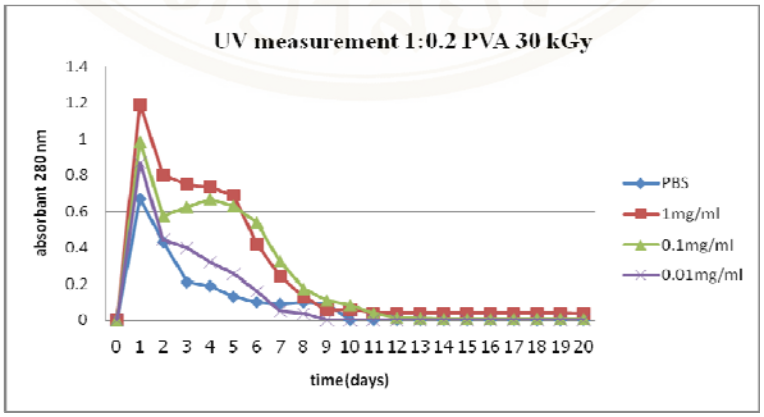
Enzymatic and negative degradation test on silk-PVA blended samples were performed at varies enzymatic condition 1, 0.1, 0.01 mg/ml. supernatant from each sample was collected and detected the protein loss using UV measurement at 280 nm. Sample in PBS was used as negative control. Figure 4.8 describes that the protein loss due to the enzyme degradation on silk-PVA sample irradiated at three doses, collected the supernatant solution. All samples showed high protein released during first day of degradation then decay rapidly on the second day. Indicating that the hydrolysis of sample was occurred at begin following by the enzyme degradation.

The enzyme activities on degrading silk-PVA in various samples are quite similar the degradation of sample immersing in PBS without enzyme and with 0.01 mg/ml protease is almost the same. They decay after 1 day to hydrolysis until 9-10

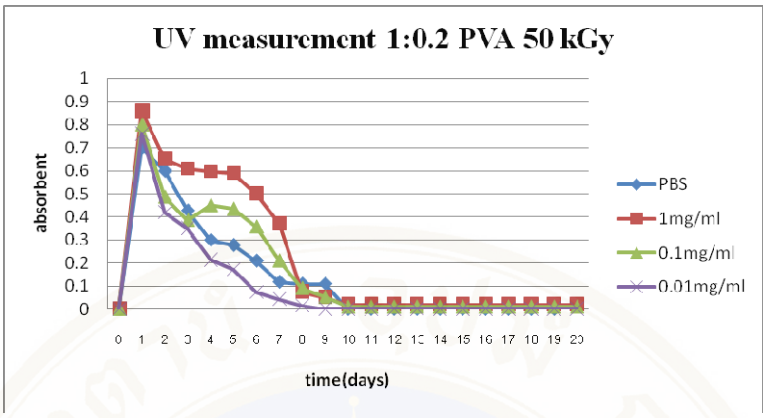
day. The protein secretion of silk-PVA in 0.1 mg/ml shows the increase in supernatant gradually after the second day until rise. The maximum at about the 5 day then decay afterward. However, the supernatant 1 mg/ml enzyme has a different activity pattern which protein seen to content at 2 day to 5 day of culturing. The decay after the 5 day comparing the absorbent protein in three set of sample irradiated at different doses, the lower irradiation dose has the highest protein release during the enzymatic action (during 4-5) and the sample irradiated at the highest irradiation dose has a lowest protein release as seen in figure 4.8(c).



(a)



(b)



(c)

Figure 4.8 UV-VIS measurement of 1:0.2 PVA (a) 10 kGy, (b) 30 kGy, (c) 50kGy

Gel electrophoresis is a technique widely used to separate proteins according to their electrophoretic mobility a function of length of polypeptide chain or molecular weight, this experiment to separate the protein molecules that degraded by protease enzyme at varies concentration around 131 to 20 kDa. The enzymatic degradation of irradiated silk-PVA hydrogel at varies enzyme concentration were shown in figure 4.9. The protein secretions from hydrogel were collected from supernatant after 5, 15, and 20 days. The results showed that the activities of enzyme decrease gradually as the time proceed. The protein band at about 30 kDa disappeared after 10 day which agrees with OD measurement in figure 4.8. The band of this protein stays at 5 day with enzyme concentration of 1 mg/ml. In fact, it can be seen with highest enzyme concentration of 1 mg/ml only. The segradation seen to be decrease especially when the irradiated dose reach to 50 kGy significantly, the sample prepared at silk-PVA 1:0.2, increasing the PVA seen to decrease the degradability as shown in figure 4.9 (f) and 4.9 (a) at 10 kGy of PVA 0.1 can be degraded very easily. These indicating that the irradiation dose and PVA content can enhance the gel formation.

M	5day			15day			20day		
	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01

M	5day			15day			20day		
	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01

a)

b)

b)

d)

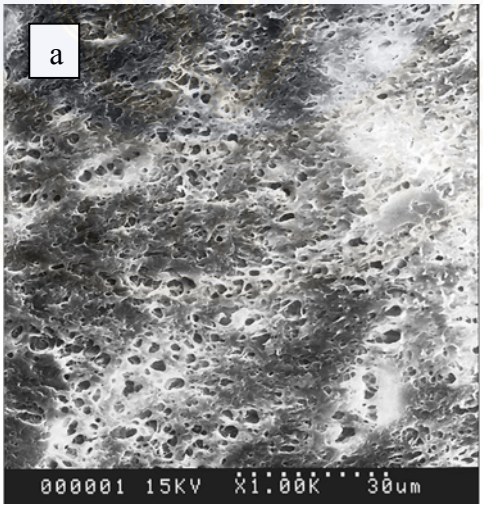
e)

f)

Figure 4.9 gel electrophoresis of hydrogel; M = marker, (a) : 10 kGy, PVA 0.1,(b) : 10 kGy, PVA 0.2, (c) : 30 kGy, PVA 0.1 (d) : 30 kGy, PVA 0.2 (e) : 50 kGy, PVA 0.1, (f) : 50 kGy, PVA 0.2, [1, 0.1, 0.01 mg/ml]

4.5 Porosity of silk-PVA hydrogel structure

The suitable silk-PVA hydrogel (1:0.01) obtained from previous was mixed with NaCl particles at different ratio (silk-PVA solution to salt particles) from 1:0.01 to 1:0.07. The hydrogel without salt showed the dense structure on the surface probably dui to the water occupancy of the hydrogel. However, the sample irradiated at 50 kGy have less pore which make them very difficult to use for artificial skin substitute in order to in area the porosity of the hydrogel



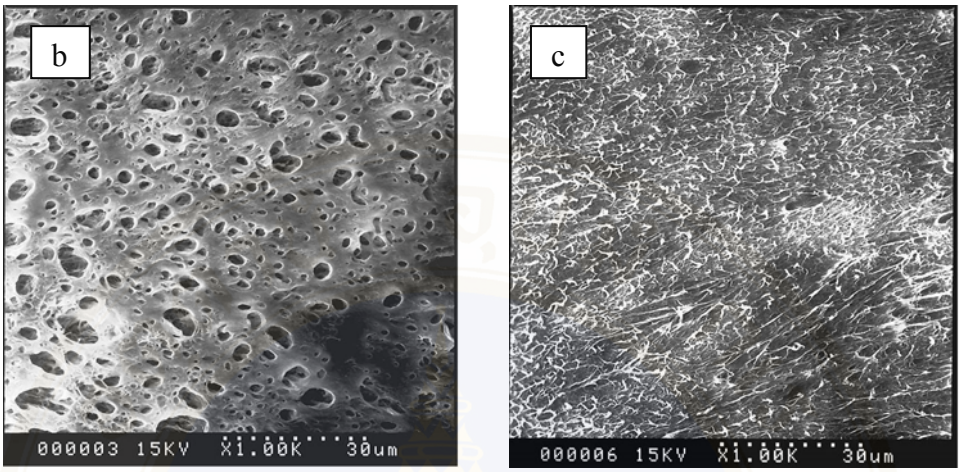


Figure 4.10 porosity of silk-PVA hydrogel structure non adding salt particles irradiated at different doses (a) 30 kGy (b) 40 kGy (c) 50 kGy (1000X)

The addition of salt particles in silk-PVA hydrogel can induce the porous structure in hydrogel as seen in figure 4.11. The SEM of silk-PVA hydrogel with salt showed the hydrogel with 1:0.01, salt has average pore size about 131 micron by increasing the amount of addition to 1:0.03, the pore size in hydrogel is also increase to 200.16 micron. These probably dui to the binding of Na^+ ion of salt particle with side structure of amino acid of silk protein make them more hydrophilic which can hold more water as show in the figure 4.11.

However, by adding salt particle higher than 1:0.03 the pore size is reduced to 96.6 micron and 98.65 micron in 1:0.05 and 1:0.07 respectively. These probably dui to the imbalance of salt protein which can destroy the pore structure as well. These can be seen in figure 4.11 c and d where the protein structure has been destroyed.

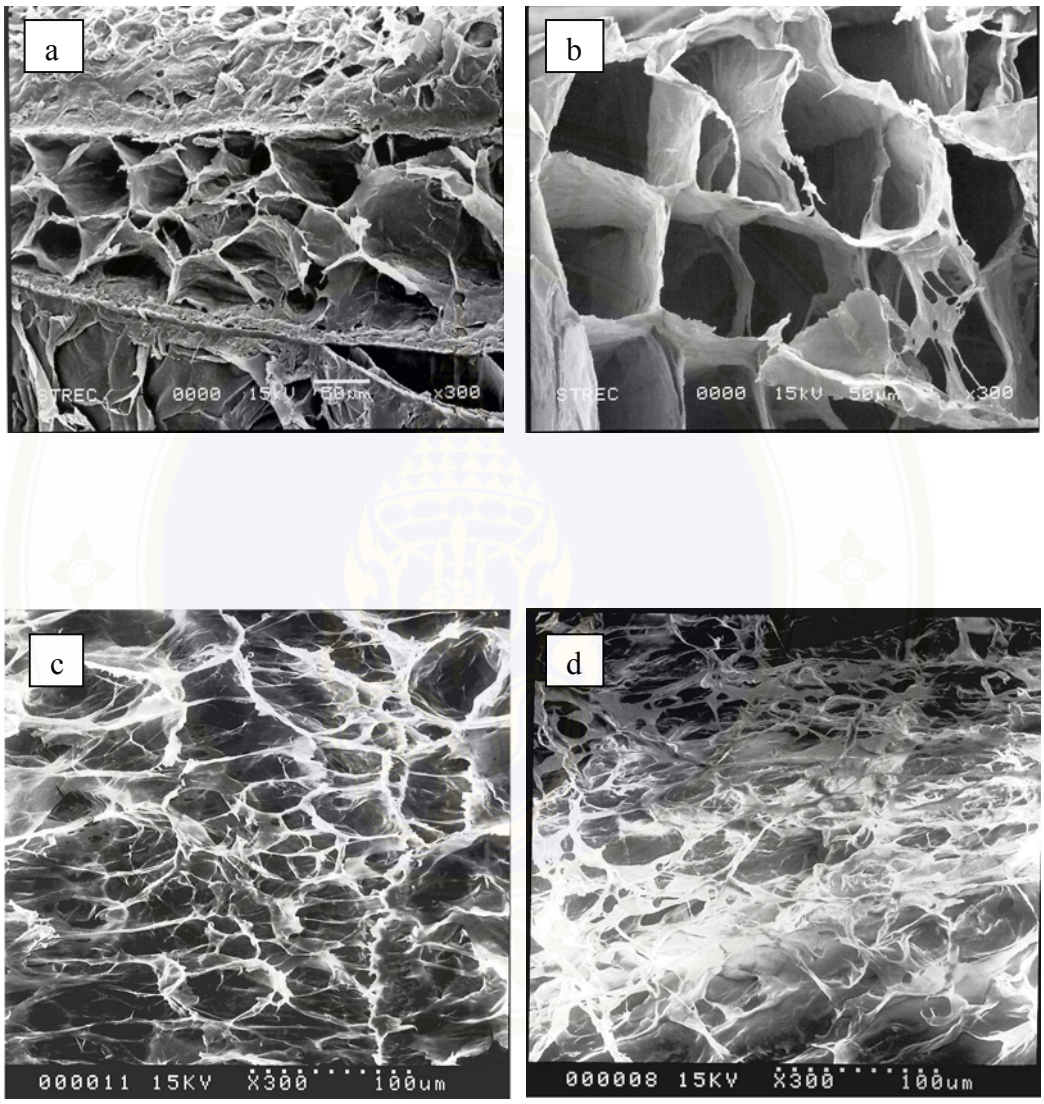


Figure 4.11 SEM of hydrrogel adding salt particle (a) 1:0.01-131 micron, (b) 1:0.03-200.16 micron, (c) 1:0.05- 96.6 micron, (d) 1:0.07-98.65 micron [300X]

4.6 Cell culture and Toxicity

In order to evaluate the silk-PVA hydrogel for artificial skin substitute, the silk-PVA at 1:0.1 molar ratio was mixed with salt at 1:0.07 ratio and irradiated with gamma ray at 30 kGy. The prepared gel was cultured in dish and the fibroblast cell was added in the culture dish.

The result of fibroblast cells on silk hydrogel were seen in figure 4.12. the number of cells on gel were immersing as the time proceed, indicating that fibroblast can grow on gel without any toxicity especially on the gel cultured for 7 days (as can seen in figure 4.12 d) most of cells can spread on the gel

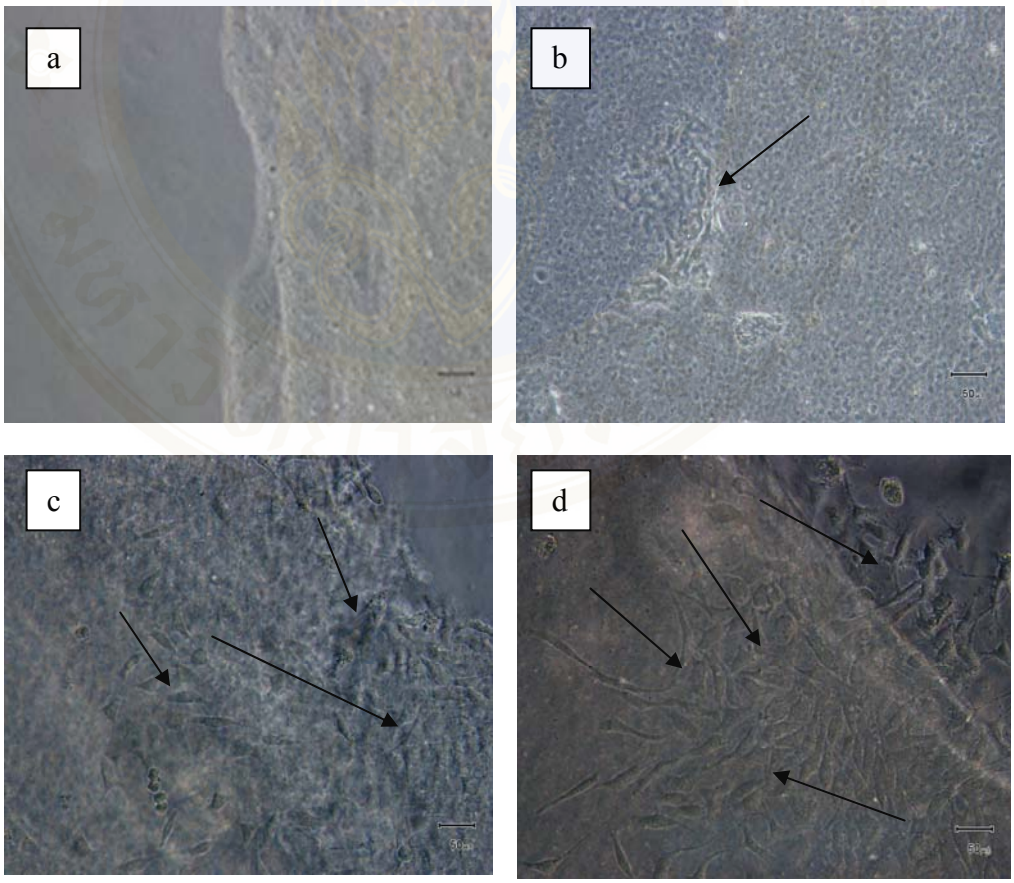


Figure 4.12 cell cultures on silk-PVA hydrogel at 1:0.1 PVA content and 1:0.07 salt particles, 30 kGy (a) 1day, (b) 3 day, (c) 5 day, (d) 7 day respectively

CHAPTER V
CONCULSION

The study of irradiated porous silk-PVA hydrogel for artificial skin substitutes can conclude that:

The amount polyvinyl (alcohol) or PVA impacts on the gelation time of silk-PVA hydrogel, the higher content of PVA induces the silk solution to gelation very fast.

The gamma ray intensity affects directly to sol-gel transition. The higher doses show that decreasing of gelation time when comparing with lower doses. The 30 kGy of gamma ray is the minimum dose that can induces the silk-PVA solution to gelation immediately.

The optimum degree of swelling is 25 kGy. The higher of gamma ray intensity shows decreasing the % degree of swelling, because its energy can induce a structure of silk-PVA hydrogel stronger. The gaps among inter-structure will decrease.

The structural change of silk-PVA hydrogel during irradiation shows shifting between random-coil to beta-sheet, ATR-FTIR is used to measure structural change. Structure is gradually shifted by increasing gamma ray intensity. The 40 and 50 kGy can be seen completely of beta-sheet structure.

In vitro degradation time of silk-PVA hydrogel rate depends on its strength of structure that induced by gamma ray and PVA content, increasing of gamma ray and PVA content induce the structure stronger. Silk-PVA hydrogel can be degraded by protease enzyme at varies concentration, the different level of % weight loss remaining and protein measuring appeared that indicating ability of degradation of concentrating enzyme from lower concentration to higher concentration significantly.

Porosity occurred when adding salt particles in a silk-PVA hydrogel, the highest ratio of salt particles quantity added that shows highest porous quantity within silk-PVA hydrogel

Cell culture and cyto-toxicity test shows that cell growing and proliferating in a silk-PVA hydrogel can ensure that the hydrogel is non toxicity and support differentiating and attaching.

Suggestion

The optimum PVA ratio should be 1:0.1 to 1:0.15, because if adding PVA more the physical property will change to liquid gel and so sticky not suitable to hydrogel.

The suitable gamma dose is range 25-30 kGy because this range is highest % degree of swelling and minimum dose that induce solution to be gelation within 30 minute of every PVA ratio content.

Unfortunately the rate of degradation should faster than 20 days and disappear simultaneously with growing of cell.

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APPENDICES

APPENDIX A

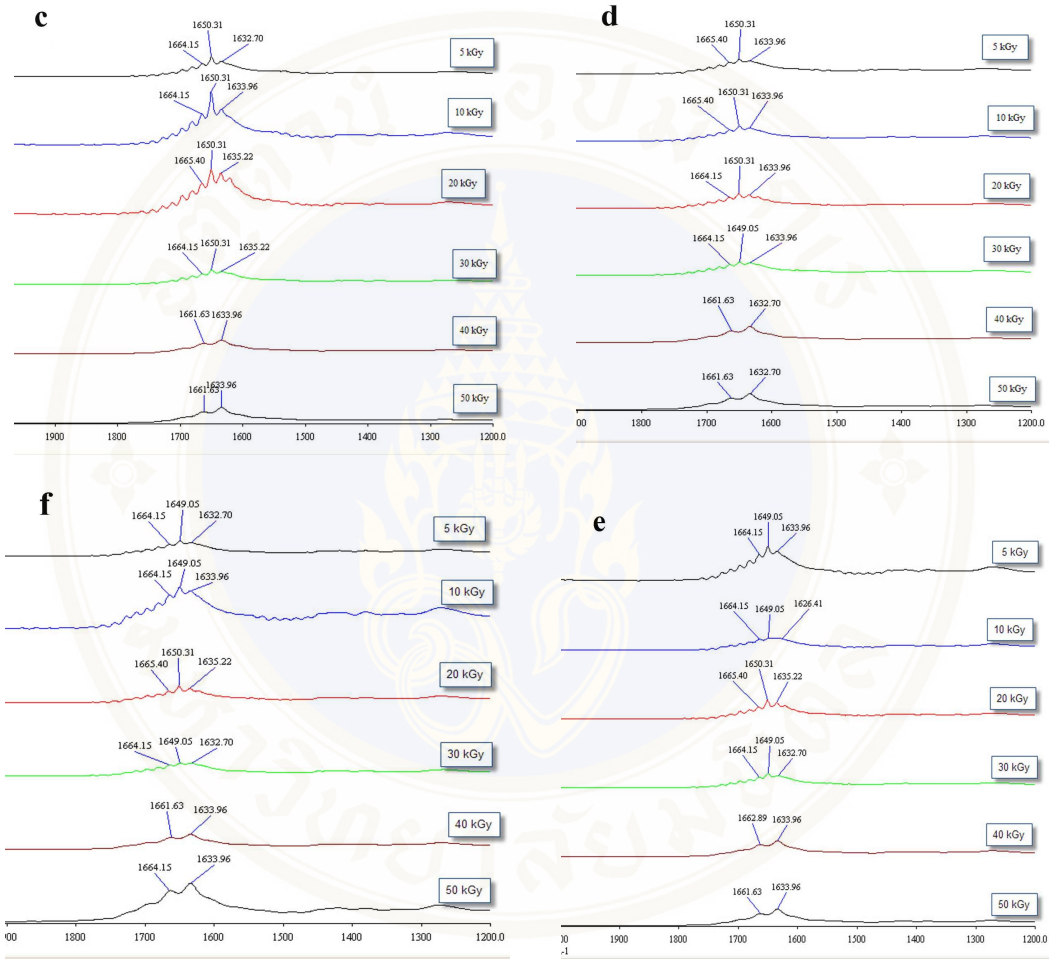


Figure A-1 FT-IR of sample (c) 1:0.1, (d) 1:0.15, (e) 1:0.2, (f) 1:0.2 of PVA content

APPENDIX B

Non-adding PVA

Radiation dose (kGy)	Alpha-helice(A.cm^{-1})	Beta-sheet(A.cm^{-1})	Beta-sheet/alpha-helice (A.cm^{-1})
5	5.5532	2.0825	0.375009
10	6.8483	2.483	0.362572
20	16.7892	3.402	0.20263
30	3.3671	2.0387	0.605477
40	5.0806	10.3313	2.03348
50	4.7068	10.4538	2.220999

1:0.05 of PVA ratio

Radiation dose (kGy)	Alpha-helice(A.cm^{-1})	Beta-sheet(A.cm^{-1})	Beta-sheet/alpha-helice (A.cm^{-1})
5	3.4186	1.7165	0.502106
10	4.3363	2.0624	0.475613
20	5.8577	2.6554	0.453318
30	2.335	1.5748	0.674433
40	3.7085	8.8691	2.39156
50	4.2014	9.8176	2.336745

1:0.1 of PVA ratio

Radiation dose (kGy)	Alpha-helice($A.cm^{-1}$)	Beta-sheet($A.cm^{-1}$)	Beta-sheet/alpha-helice ($A.cm^{-1}$)
5	5.2159	2.766	0.530302
10	6.475	3.2917	0.508371
20	3.8811	2.3129	0.595939
30	3.0082	1.2977	0.431388
40	3.209	8.2394	2.567591
50	3.6632	9.2121	2.514769

1:0.15 of PVA ratio

Radiation dose (kGy)	Alpha-helice($A.cm^{-1}$)	Beta-sheet($A.cm^{-1}$)	Beta-sheet/alpha-helice ($A.cm^{-1}$)
5	2.4748	1.3562	0.548004
10	3.1473	1.734	0.550948
20	3.3048	2.0798	0.629327
30	2.3414	1.3607	0.581148
40	4.0937	10.5408	2.574883
50	3.8	9.705	2.553947

1:0.2 of PVA ratio

Radiation dose (kGy)	Alpha-helice($A.cm^{-1}$)	Beta-sheet($A.cm^{-1}$)	Beta-sheet/alpha-helice ($A.cm^{-1}$)
5	2.9434	1.6194	0.55018
10	1.4402	1.397	0.970004
20	1.3031	1.5302	1.174277
30	3.0816	1.5891	0.515674
40	3.8961	8.676	2.226842
50	3.6546	10.0538	2.750999

1:0.25 of PVA ratio

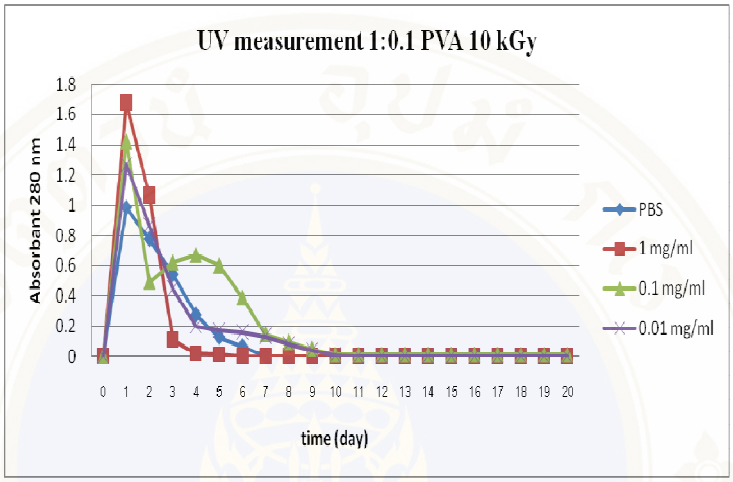
Radiation dose (kGy)	Alpha-helice($A.cm^{-1}$)	Beta-sheet($A.cm^{-1}$)	Beta-sheet/alpha-helice ($A.cm^{-1}$)
5	2.4677	1.4836	0.601208
10	2.1131	2.0281	0.959775
20	3.844	1.9424	0.505307
30	1.4093	1.1523	0.81764
40	3.5171	7.681	2.183902
50	3.2384	7.4013	2.28548

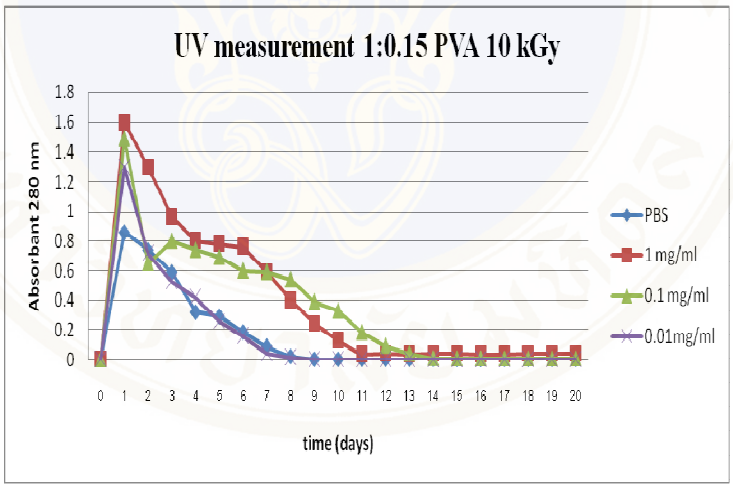
1:0.3 of PVA ratio

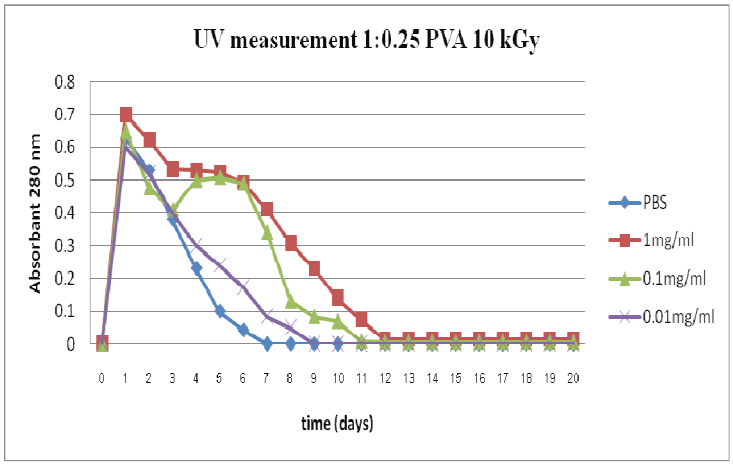
Radiation dose (kGy)	Alpha-helice($A.cm^{-1}$)	Beta-sheet($A.cm^{-1}$)	Beta-sheet/alpha-helice ($A.cm^{-1}$)
5	1.7158	1.5894	0.926332
10	2.2598	2.2645	1.00208
20	2.5399	1.7864	0.703335
30	2.0616	1.4594	0.707897
40	3.4072	8.4695	2.485765
50	3.2038	6.1335	1.914445

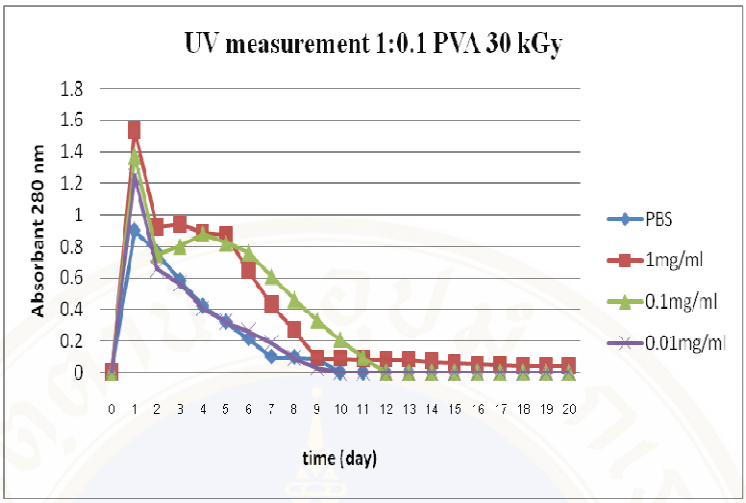
Table B-1 the beta-sheet to random-coil ratio ($A.cm^{-1}$) of silk-PVA hydrogel at varies PVA ratio

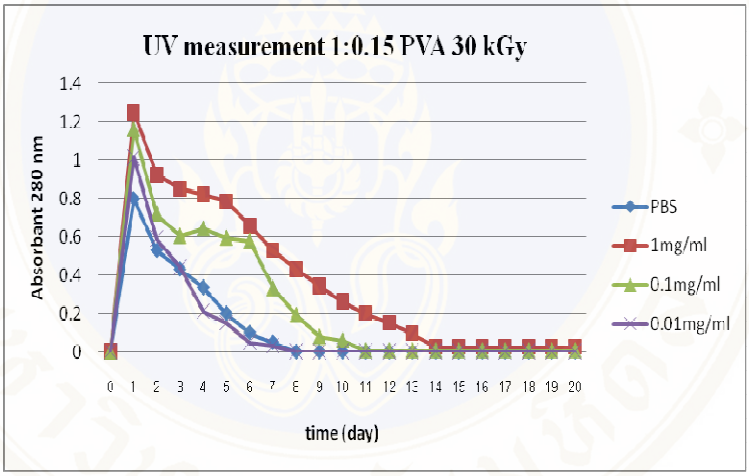
APPENDIX C

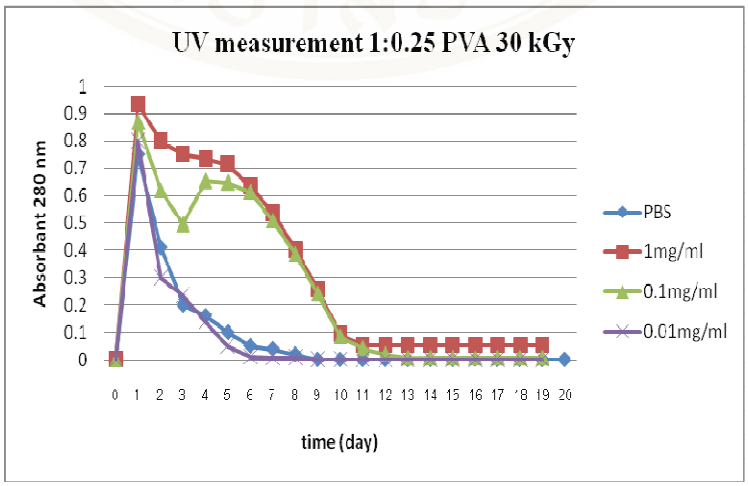


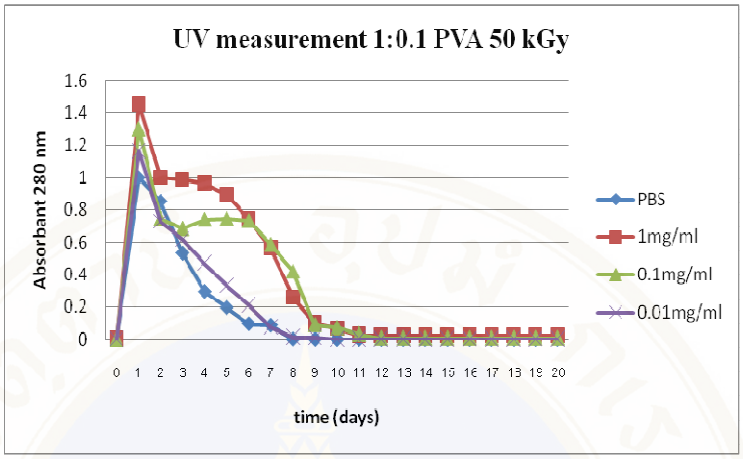


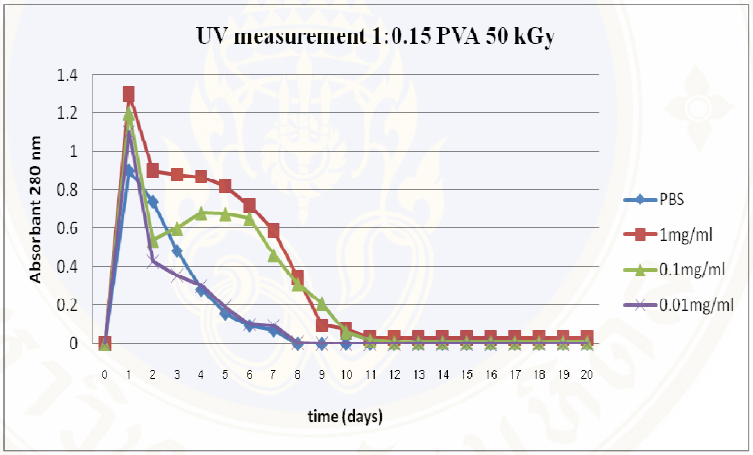












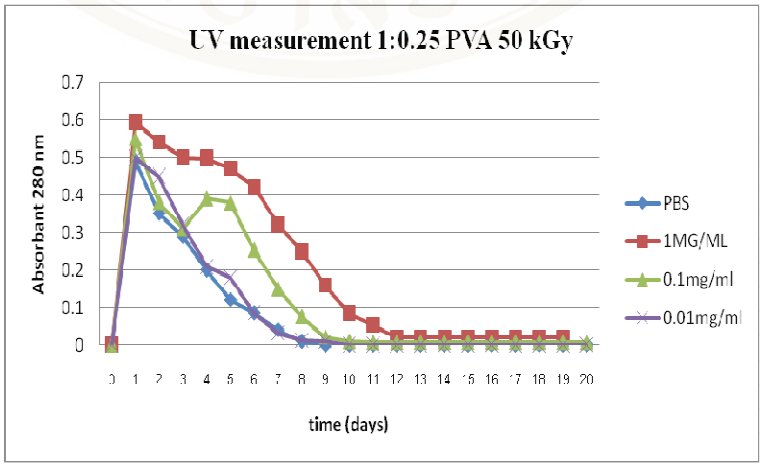
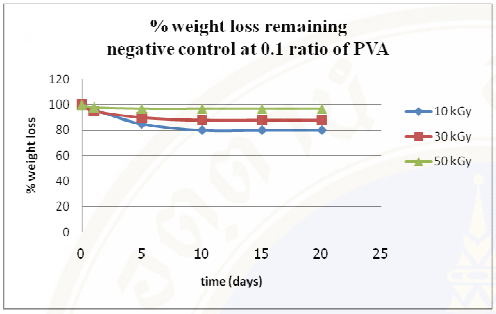
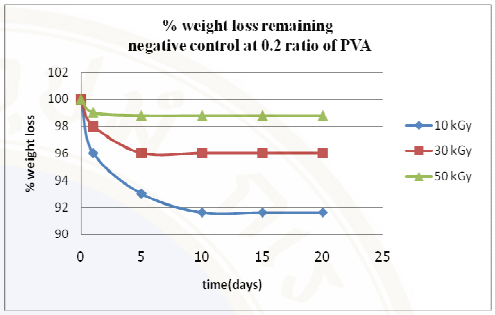
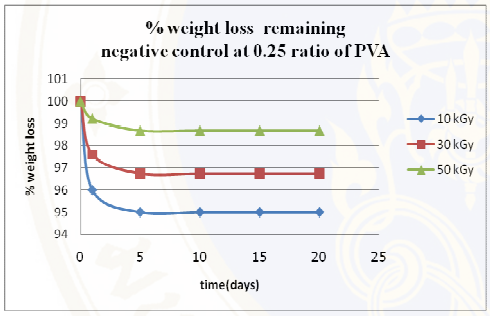


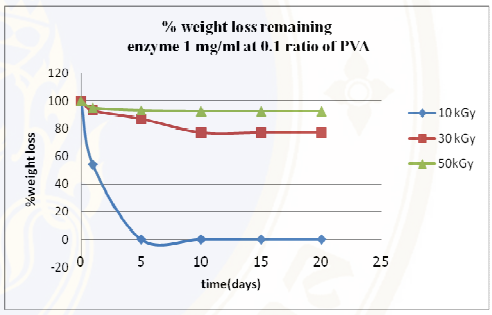
Figure c-1 UV measurement of hydrogel at 280 nm

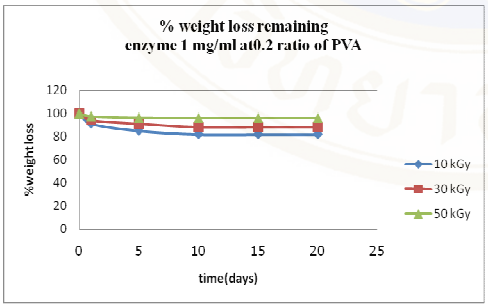
APPENDIX D

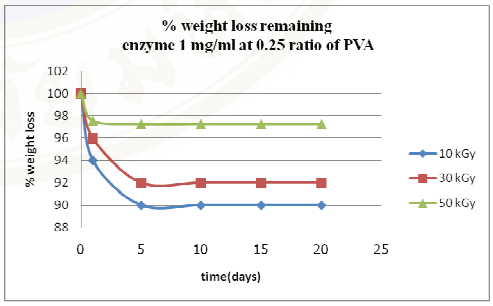












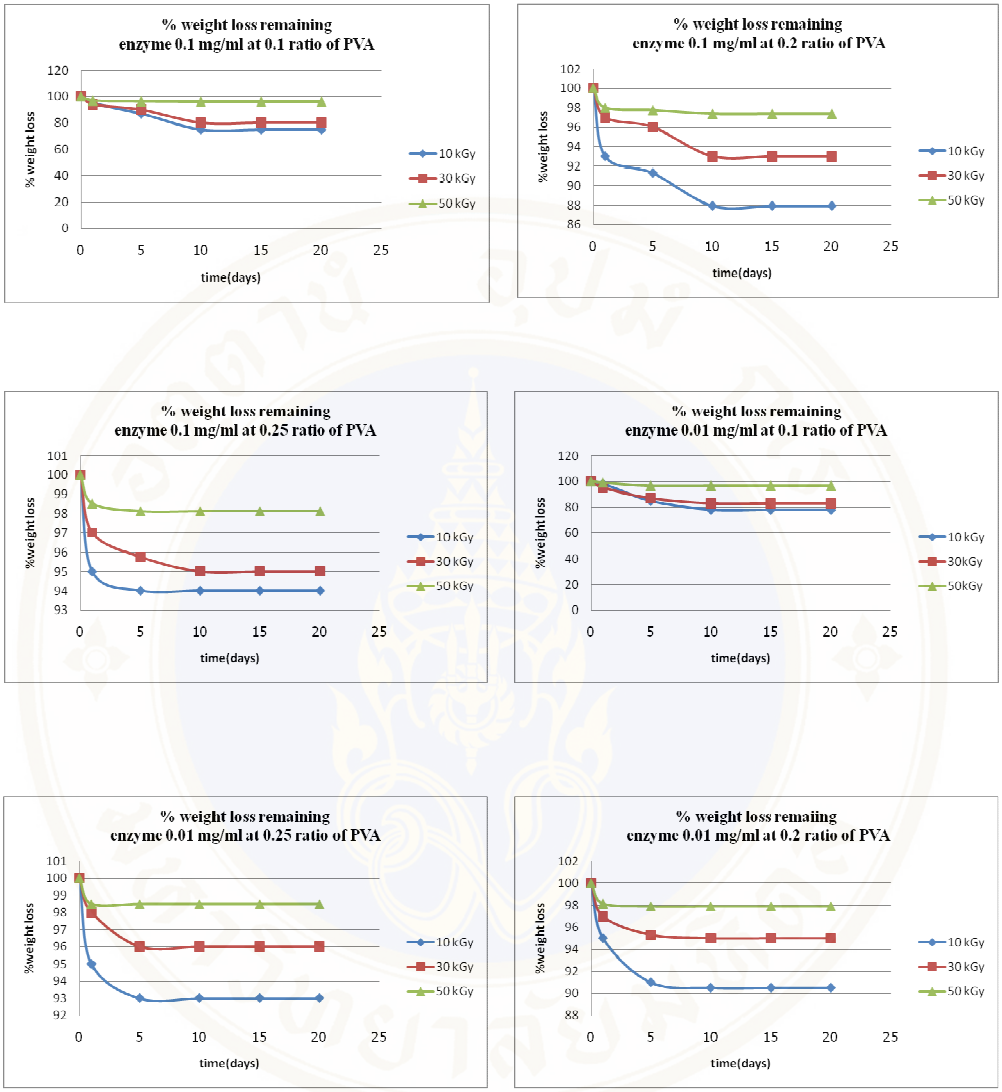
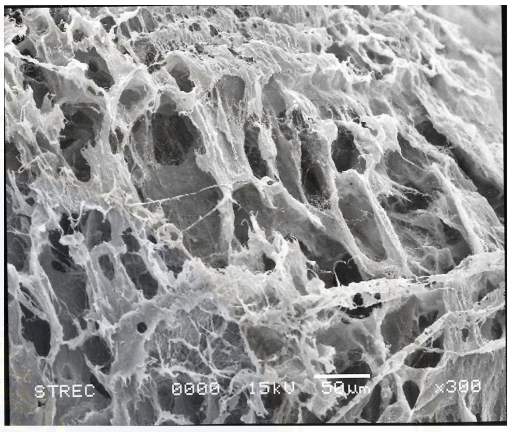


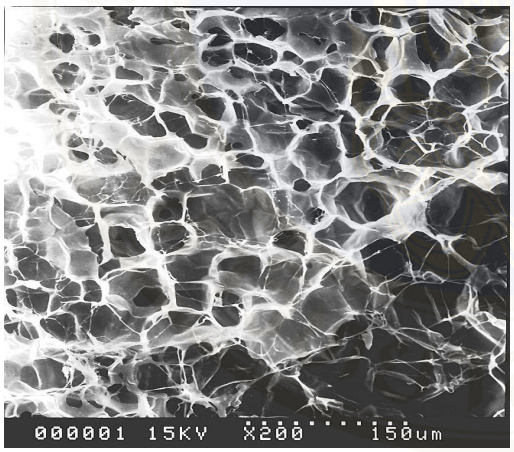
Figure C-1 %weight loss remaining of hydrogel during degradation

APPENDIX E

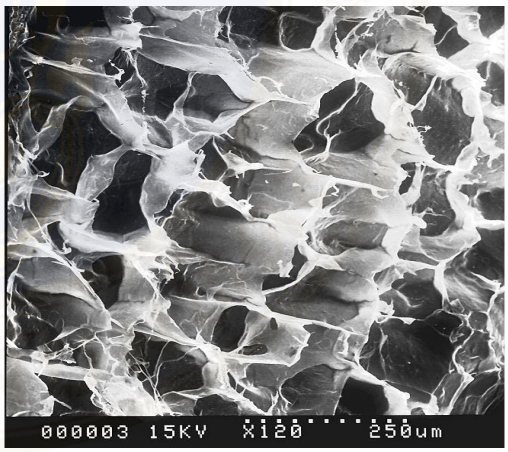
30 kGy 1:0.05 of salt paticles



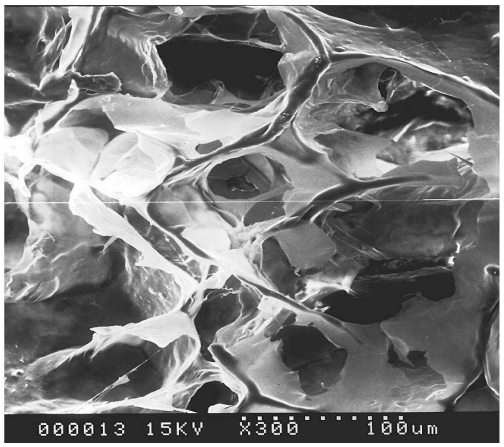
30 kGy 1:0.07 of salt paticle

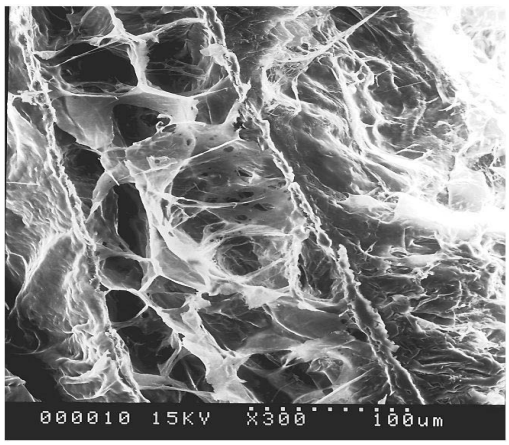


40 kGy 1:0.01 of salt particles



40 kGy 1:0.05 of salt paticles





50 kGy 1:0.01 of salt paticles 50 kGy 1:0.03 of salt paticlesFigure D-1 SEM of porous hydrogel adding salt particles at varies ratios

BIOGRAPHY

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PLUBICATION	A Study of Irradiated Silk Fibroin-PVA Hydrogel for Artificial Skin Substitutes, Intavisade,P. and Oonkhanond, B., journal of metals, materials and minerals, Vol. 20, No. 3 (Dec 2010)