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EFFECTS OF GELATIN CONJUGATION AND HYDROXYAPATITE DEPOSITION ON THAI SILK FIBROIN SCAFFOLDS

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Thesis Title	EFFECTS OF GELATIN CONJUGATION AND HYDROXYAPATITE DEPOSITION ON THAI SILK FIBROIN SCAFFOLDS
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ชัชวรรณ ตริตานิภากุล : ผลกระทบของการคอนจูเกตเจลาตินและการสะสมไฮดรอกซีอปาไทต์บนโครงเลี้ยงเซลล์ที่ทำจากไฟโบรอินไหมไทย. (EFFECTS OF GELATIN CONJUGATION AND HYDROXYAPATITE DEPOSITION ON THAI SILK FIBROIN SCAFFOLDS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ศิริพร ดำรงค์ศักดิ์กุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.รัฐ พิชญางกูร, 95 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลกระทบจากการคอนจูเกตเจลาติน และการสะสมไฮดรอกซีอปาไทต์ที่มีต่อสมบัติทางกายภาพและทางชีวภาพของโครงเลี้ยงเซลล์ไฟโบรอินไหมไทยเพื่อใช้ในงานวิศวกรรมเนื้อเยื่อกระดูก โดยโครงเลี้ยงเซลล์ไฟโบรอินไหมไทยถูกเตรียมด้วยวิธีการกำจัดเกลือออก โครงสร้างสัณฐานของโครงเลี้ยงเซลล์แสดงให้เห็นว่าโครงเลี้ยงเซลล์มีพื้นผิวเรียบ และมีรูพรุนเชื่อมต่อกัน โครงเลี้ยงเซลล์ไฟโบรอินไหมไทยถูกปรับปรุงพื้นผิวด้วยการคอนจูเกตกับเจลาติน และสะสมไฮดรอกซีอปาไทต์บนพื้นผิวของโครงเลี้ยงเซลล์ด้วยวิธีการแช่สลับในสารละลายแคลเซียมคลอไรด์ และสารละลายโซเดียมไฮโดรเจนฟอสเฟต ความเข้มข้นของสารละลายเจลาตินที่ใช้ในการปรับปรุงพื้นผิวโครงเลี้ยงเซลล์ไฟโบรอินไหมไทยมีผลต่อปริมาณเจลาตินที่มาคอนจูเกตบนโครงเลี้ยงเซลล์ ลักษณะสัณฐานของโครงเลี้ยงเซลล์ และการเจริญเติบโตของเซลล์บนโครงเลี้ยงเซลล์ ภายหลังจากกระบวนการแช่สลับในสารละลายแคลเซียมคลอไรด์ และสารละลายโซเดียมไฮโดรเจนฟอสเฟตพบว่ามีไฮดรอกซีอปาไทต์มาสะสมอยู่บนพื้นผิวของโครงเลี้ยงเซลล์ส่งผลให้โครงเลี้ยงเซลล์มีพื้นผิวขรุขระ และมีความพรุนลดลง โดยโครงเลี้ยงเซลล์ที่แช่ในสารละลายแคลเซียมคลอไรด์ก่อน แล้วตามด้วยสารละลายไฮโดรเจนฟอสเฟตจำนวน 4 รอบ เป็นเวลา 30 นาทีในสารละลายแต่ละชนิดเป็นกระบวนการแช่สลับที่ทำให้โครงสร้างของโครงเลี้ยงเซลล์มีความพรุน และความแข็งแรงที่เหมาะสม จากผลการทดสอบด้วยเทคนิคเทอร์โมกราวิเมตริกอนาไลซิสชี้ให้เห็นว่าปริมาณไฮดรอกซีอปาไทต์จากการแช่ในสารละลายคลอไรด์และสารละลายฟอสเฟตสลับกันจำนวน 4 รอบ บนโครงเลี้ยงเซลล์มีค่าประมาณ 52% อัตราส่วนแคลเซียมต่อฟอสเฟตของโครงเลี้ยงเซลล์ไฟโบรอินไหมไทย และโครงเลี้ยงเซลล์ไฟโบรอินไหมไทยที่มีการปรับปรุงพื้นผิวด้วยเจลาติน และมีการสะสม ไฮดรอกซีอปาไทต์จำนวน 4 รอบมีค่าเท่ากับ 1.71 และ1.51 ตามลำดับ ผลการเลี้ยงเซลล์ต้นกำเนิดไขกระดูกในระดับห้องปฏิบัติการพบว่าโครงเลี้ยงเซลล์ไฟโบรอินไหมไทยที่มีการปรับปรุงพื้นผิวด้วยเจลาตินที่มีความเข้มข้นของสารละลายเจลาติน 1.0% โดยน้ำหนัก มีประสิทธิผลในการชักนำให้เกิดการเปลี่ยนแปลงไปเป็นกระดูกได้ดีเช่นเดียวกับโครงเลี้ยงเซลล์ที่มีการสะสมไฮดรอกซีอปาไทต์ ซึ่งเห็นได้จากปริมาณแอลคาไลน์ฟอสฟาเตส ปริมาณแคลเซียม และลักษณะสัณฐานของเซลล์บนโครงเลี้ยงเซลล์ ผลการศึกษาแสดงให้เห็นว่าโครงเลี้ยงเซลล์ไฟโบรอินไหมไทยที่มีการปรับปรุงพื้นผิวด้วยเจลาตินและสะสมไฮดรอกซีอปาไทต์มีสมบัติทางกายภาพ และทางชีวภาพที่เหมาะสมสำหรับประยุกต์ใช้ในงานวิศวกรรมเนื้อเยื่อ

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา....วิศวกรรมเคมี....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2552.....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4970275721 : MAJOR CHEMICAL ENGINEERING

KEY WORD: SILK FIBROIN / GELATIN / HYDROXYAPATITE /SCAFFOLD / BONE MARROW-DERIVED STEM CELLS / TISSUE ENGINEERING

SHATSHAWAN TRITANIPAKUL : EFFECTS OF GELATIN CONJUGATION AND HYDROXYAPATITE DEPOSITION ON THAI SILK FIBROIN SCAFFOLDS. THESIS ADVISOR : ASSOC.PROF.SIRIPORN DAMRONGSAKKUL, Ph.D., THESIS CO-ADVISOR : ASST. PROF RATH PICHYANGKURA, Ph.D., 95 pp.

This research aimed to investigate the effects of gelatin conjugation and hydroxyapatite deposition on the physical and biological properties of Thai silk fibroin scaffolds for bone tissue engineering. Thai silk fibroin scaffolds were prepared via salt-leaching method. The morphology of scaffolds showed smooth surface and interconnected porous network. Surface of silk fibroin scaffolds were modified by gelatin conjugation and hydroxyapatite deposition by alternate soaking in calcium chloride and disodium hydrogenphosphate solutions. The different concentration of gelatin solution was found to influence the amount of gelatin conjugated onto Thai silk fibroin scaffolds, the morphology of the scaffolds and the cell proliferation on the scaffolds. After alternate soaking, hydroxyapatite was observed on the pore surface of scaffolds leading to the rough surface of porous structure and less pore volume. The scaffolds obtained from 4 cycles of alternate soaking in calcium chloride and disodium hydrogenphosphate solutions alternately with the soaking time for 30 minutes in each solution showed suitably porous morphology and compressive modulus. The results on TGA indicated that hydroxyapatite deposited after four cycles of alternate soaking in the scaffolds were about 52%. The Ca/P ratio of hydroxyapatite/Thai silk fibroin and hydroxyapatite/ conjugated gelatin/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking were 1.71 and 1.51, respectively. The results on *in vitro* culture using bone-marrow derived mesenchymal stem cells showed that conjugated gelatin/Thai silk fibroin scaffold prepared from 1.0wt% gelatin concentration effectively enhanced osteogenic differentiation similar to the scaffolds containing hydroxyapatite as evaluated by alkaline phosphatase activity, calcium content and the morphology of cultured cell on the scaffold surface. The results on the physical and biological properties of hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds markedly indicated that the scaffolds had a high potential to be applied in tissue engineering.

Department :...Chemical Engineering…Student's signature……..…… .

Field of study :..Chemical Engineering..Thesis advisor's signature……..…

Academic year :… 2009……..Thesis co-advisor's signature……..……...…...

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INTRODUCTION

1.1 Background

Bone autografts and allografts have been employed for traditional healing of bone defects. The disadvantages of these treatments are the limitation of donor sites and the antigenicity of host tissues. As a result, the development and the uses of bone substitutes produced for artifical and natural materials are of interest. Commercial bone substitutes are mainly composed of ceramic and titanium. However, these bone substitutes has a limit on the rate of bone remodeling. Many research interests have focused on the utilization of natural biodegradable materials, eg. collagen and silk fibroin, as scaffolds due to their biocompatible and biodegradable characteristics. These includes the development of three-dimensional scaffolds for bone cells or bone substitutes [1, 2, 3].

In the process of bone regeneration, three dimensional scaffolds are required to promote cell, tissue attachment and growth. They must be biocompatible, osteoconductive and can be degraded at an appropriated rate. Sufficient mechanical properties of the scaffolds are also necessary to support tissue function and integration. Some protein-based biomaterials such as collagen is known to be the most promising materials in tissue engineering applications because of their biological similarities to natural proteins. However, its use as bone implant materials is limited due to its poor mechanical properties. In contrast, silks offer distinguishing mechanical properties that are tailorable, along with slow degradability to permit appropriate time for remodeling. [3]

Silk fibroin is a major constituent of raw silk fiber. It has been used commercially as biomedical sutures for centuries. Apart from its impressive mechanical properties, its also has environmental stability, biocompatibility, controlled proteolytic biodegradability, low antigenicity, morphological flexibility

and the ability for amino acid side chain modification to immobilize growth factors. Furthermore, silk fibroin-based scaffolds demonstrate the feasibility of silk-based implants with engineered bone for the regeneration of bone tissues [4]. Thai silk is one of *Bombyx mori* silkworms. Characteristics of cocoon Thai silk are its yellow color and coarse filament. Thai silk is locally available and well known in textile industry for a long time. In the recent years, there are a few reports of Thai silk scaffolds for tissue engineering such as preparation of electrospun silk fibroin fiber mats as bone scaffolds [5].

Previous work of our group by Chamchongkaset [6] showed that “Nangnoi Srisaket 1” Thai silk fibroin could be fabricated into salt-leaching scaffolds. Thai silk fibroin scaffolds were modified by gelatin conjugating as gelatin contains arginine-glycine-aspartic acid (RGD)-like sequence that promotes cell adhesion and migration [7]. Furthermore, hydroxyapatite, which is reported to enhance mineralization and bone formation [8], was deposited into the scaffolds by alternating soaking method. Gelatin conjugating was found to enhance mechanical property and favorable to osteoblast-like cell proliferation. However, *in vitro* biological tests of the developed scaffolds have not been fully investigated in details yet.

It is therefore the aim of this research to focus on the effects of gelatin conjugation and hydroxyapatite deposition on the physical and biological properties of Thai silk fibroin scaffolds as bone substitute. The effects of the concentration of gelatin solution and parameters of alternative soaking process including soaking time in each solution, alternate soaking step and cycles of alternate soaking to deposit hydroxyapatite on the properties of scaffolds will be investigated. The morphology, compression modulus, *in vitro* biocompatibility using rat bone marrow mesenchymal stem cells (MSCs) will be systematicly examined.

1.2 Objectives

1.2.1 To investigate the effects of gelatin conjugation and hydroxyapatite deposition on Thai silk fibroin scaffolds.

1.2.2 To investigate the physical and biological properties of Thai silk fibroin scaffolds.

1.3 Scopes of Research

1.3.1 Prepare Thai silk fibroin scaffolds via salt-leaching method from cocoons of “Nangnoi srisaket1” silkworm.

1.3.2 Prepare hydroxyapatite/Thai silk fibroin and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds (11mm in diameter and 2mm in height). Parameters to be investigated are

1.3.2.1 Concentration of gelatin : 0, 0.5, 1, 1.5 wt%.

1.3.2.2 Soaking time in each solution : 10, 20, 30 min.

1.3.2.3 Cycles of alternate soaking : 0, 4, 6, 8 cycles.

1.3.2.4 Alternate soaking steps : First immersing in $CaCl_2$ solution following by Na_2HPO_4 solution, First immersing in Na_2HPO_4 solution following by $CaCl_2$ solution.

1.3.3 Characterize the physical properties of hydroxyapatite/Thai silk fibroin and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds including:

1.3.3.1 Morphology by scanning electron microscope (SEM).

1.3.3.2 Compression modulus

1.3.3.3 wt% of conjugated gelatin and deposited hydroxyapatite in each type of scaffold.

1.3.3.4 Thermal properties

1.3.3.5 Elemental analysis

1.3.4 Characterize the biological properties of hydroxyapatite/Thai silk fibroin and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds using bone marrow stem cell (MSCs) including:

1.3.4.1 Cell attachment at 6 hours and proliferation at 72 and 120 hours by DNA assay.

1.3.4.2 Cell differentiation at 1, 8, 15, 22 and 29 days by DNA assay, ALP assay and calcium assay.

1.3.4.3 Cell migration and morphological observation.

CHAPTER II

RELEVANT THEORY AND LITERATURE REVIEW

2.1 Relevant theory

2.1.1 Biomaterials

There are many biomaterials, such as natural and synthetic polymers, which have high potential as biocompatible and biodegradable materials for scaffolding. Several biomaterials including silk, gelatin and hydroxyapatite are introduced for the fabrication of scaffolds in this study.

2.1.1.1 Silk [9, 10, 11]

Silk is naturally occurring protein fiber produced by *Lepidoptera larvae* such as silkworms, spiders and mites. The most widely interested silks are silkworm *Bombyx Mori* and spider *Nephila clavipes* due to their properties utilizable in high quality textiles and biomedical field. Silk is defined as fibrous protein polymers containing highly repetitive sequences of amino acids. Silk protein is synthesized in the silk glands of the silkworm. It is secreated and stored in the lumen thereafter its spinned into fibers.

Silk fiber from *Bombyx mori* consists of silk sericin (about 25 wt%), silk fibroin (about 75%), fat and oil (0.5 – 1 wt%) and raw silk filament (1 – 1.4 wt%). Structure of silk fiber as shown in Figure 1.

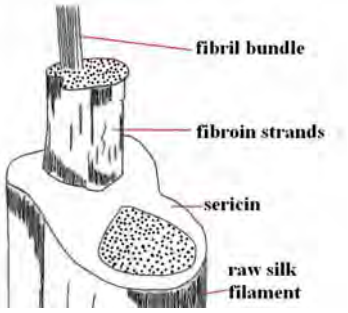

Figure 2.1 Structure of silk fiber [12]

Silk sericin

Sericin is the water-soluble protein that binds fibroin fibers together. It is a yellow, brittle, and inelastic substance. Silk sericin can be removed during the degumming process using soap or acid.

Silk fibroin

A major constituent of raw silk fiber is silk fibroin. Fibroin is not water-soluble protein. Its structure is composed of layers of antiparallel beta pleated sheets (Figure 2.2) which run parallel to the silk fiber axis. Silk fibroin fibers are about 10 – 25 mm in diameter and consist of two proteins: light chain (~26k Da) and heavy chain (~390k Da) and linked by a single disulfide bond. The disulfide linkage between the Cys-c20 (20^{th} residue from the carboxyl terminus) of the heavy chain and Cys-172 of the light chain holds the fibroin together and a 25k Da glycoprotein (P25) is noncovalently linked to these proteins. The heavy chain consists of 12 repetitive regions called crystalline regions and 11 non repetitive interspaced regions called amorphous regions. The composition of silk fibroin consists primarily of 43% glycine, 30% alanine and 12% serine.

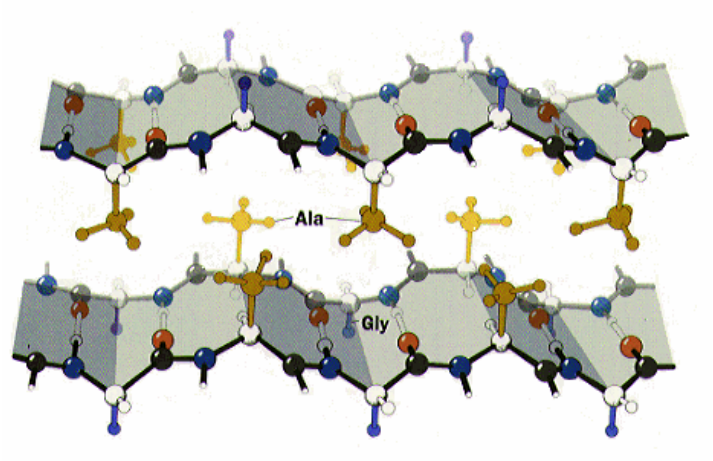

Figure 2.2 Structure of fibroin [13]

The primary structure of *Bombyx mori* silk fibroin may be approximately divided into four regions as shown in Figure 2.3. Region 1 is the highly repetitive GAGAGS sequence which constitutes the crystalline part of the fibroin (94% of total chain). Region 2 is the relatively less repetitive GAGAGY and/or GAGAGVGY sequences consisting of the semi-crystalline parts. Region 3 is GAGAGSGAAS.

Region 4 is the amorphous part containing charged and aromatic residues. (G is glycine, A is alanine, S is serine V is valine and Y is tyrosine).

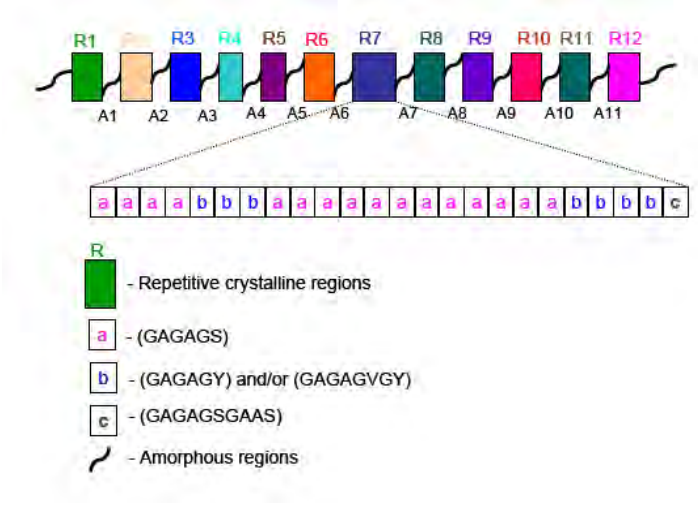

Figure 2.3 Schematic representation of the primary structure of *B. mori* silk [14].

Thai silk

Thai silk is one of *Bombyx mori* silkworms. Thai silk is domesticly produced in the northern and north-eastern parts of Thailand mainly for textile industry. Yellow color and coarse filaments are the distinct characteristics of Thai silk. Thai silk fibers also contain more silk gum (up to 38%) than normal *Bombyx mori* silk (20 – 25%). There are about 28 species of Thai silk such as Nangnoi srisaket 1 and Nangline including other species of blended-Thai silk such as blended-sakolnakorn and blended-ubonratchathani 60-35 (lotus).

- Nangnoi srisaket 1

This specie is easily cultivated. The life cycle is short (about 18 days). The color of the cocoon is dark yellow.

- Nangline

This specie is easily degummed. The color of cocoon is dark yellow. The weight of cocoon is 0.68 – 1.64g. The length of silk fiber is about 311 m/cocoon.

- Blended-Ubonratchathani 60-35

This specie is blended between Ubonratchathani 60 and Nangnoi srisaket 1. The color of cocoon is yellow. The weight of cocoon is 1.4g. The length of silk fiber is about 519 m/cocoon.

Amino acid compositions of silk fibroin

Silk fibroin is composed of 18 amino acids (Table 2.1). The isoelectric point is around 5.

Table 2.1 Amino acid compositions of *Bombyx mori* silk fibroin [14].

Amino Acid	Symbol	Charge	Hydrophobic/ Hydrophilic	Amount (g/100 g silk fibroin)
Alanine	Ala	neutral	hydrophobic	32.4
Glycine	Gly	neutral	hydrophilic	42.8
Tyrosine	Tyr	neutral	hydrophilic	11.8
Serine	Ser	neutral	hydrophilic	14.7
Aspartate	Asp	negative	hydrophilic	1.73
Arginine	Arg	positive	hydrophilic	0.90
Histidine	His	positive	hydrophilic	0.32
Glutamate	Glu	negative	hydrophilic	1.74
Lysine	Lys	positive	hydrophilic	0.45
Valine	Val	neutral	hydrophobic	3.03
Leucine	Leu	neutral	hydrophobic	0.68
Isoleucine	Ile	neutral	hydrophobic	0.87
Phenylalanine	Phe	neutral	hydrophobic	1.15
Proline	Pro	neutral	hydrophobic	0.63
Threonine	Thr	neutral	hydrophilic	1.51
Methionine	Met	neutral	hydrophobic	0.10
Cysteine	Cys	neutral	hydrophobic	0.03
Trytophan	Trp	neutral	hydrophilic	0.36

Properties of silk

- Silk is an excellent combination of lightweight. Its density is 1.3 g/cm^3.
- Silk is one of the strongest and toughest natural fibers. Its strength is up to 4.8 GPa.
- Silk is thermally stable up to 250ºC, allowing processing over a wide range of temperature.
- Silk is a poor conductor of electricity.
- Silk is biocompatible and biodegradable. Its protein composition makes it compatible with human body.

Applications of silk

Silk is used in many applications including textile industry, cosmetics and medical applications. It is well known that silk thread has been used for surgery. In other medical fields, gauze pads, and bandaged for dermatological disorders made from silk have been commercialized. Furthermore, silk continues to find new uses such as artifical skin, blood vessels, and tendons. A nonwoven cloth called silk sheet helps to keep fruit and vegetables fresh, with a significant economic benefit. Attempts are now being made to use silk to clean rivers polluted by household and industrial waste water. The pollutants are absorbed and broken down with bacteria cultured in gaps between scrap cocoons. Examples of silk applications are shown in Figure 2.4.

Figure 2.4 Applications of silk [15].

2.1.1.2 Gelatin [7, 16]

Gelatin is a natural polymer derived from collagen. Its random coil structure can be readily soluble in water. Gelatin forms thermo-reversible gel, with the gel point below 30°C (depending on viscosity). Gelatin is commonly used for pharmaceutical and medical application because of its biodegradability and biocompatibility in physiological environments.

Gelatin can be divided into 2 types depending on the production process as shown in Figure 2.5.

Acidic gelatin

Acidic gelatin (Type B gelatin) is derived from alkaline treatment of collagen. The alkaline process, targets the amide groups of asparagines and glutamine, and hydrolyses them into carboxyl groups, thus converting many of these residues to aspartate and glutamate. The alkaline processed gelatin possesses a greater proportion of carboxyl groups. It contains negatively charged and lowering its isoelectric point (IEP). Acidic gelatin has IEP about 5.0.

Basic gelatin

Basic gelatin (Type A gelatin) is derived from acid treatment of collagen. Basic gelatin produced from a milder condition, the products consist of higher amount of amino group. It contains more positive charges than that of type B gelatin. Type A gelatin has IEP at 9.0.

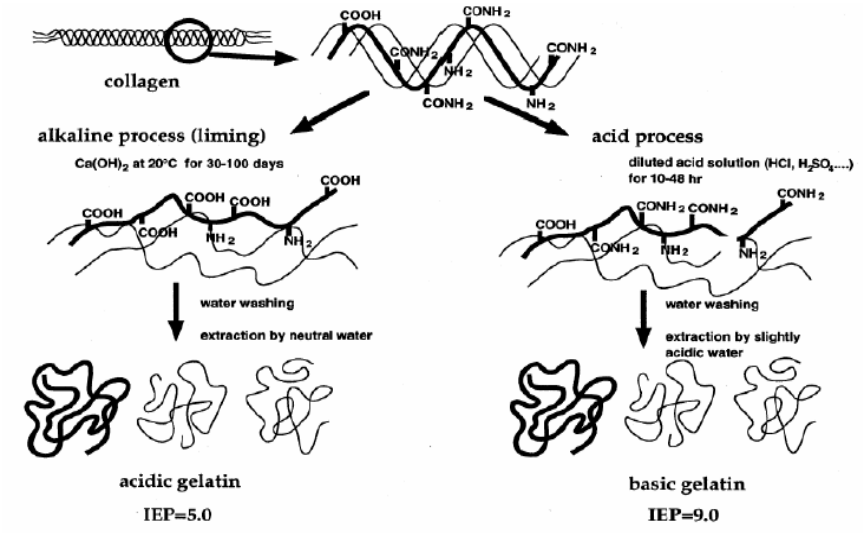

Figure 2.5 Preparation processes for acidic and basic gelatins from collagen [7].

Specifications for type A and B gelatin are summarized in Table 2.

Table2.2 Specifications for type A and B gelatin [17].

Property	Type A	Type B
pH	3.8 – 5.5	5.0 -7.5
Isoelectric point (pI)	7.0 – 9.0	4.7 – 5.4
Gel strength (bloom)	50 – 300	50 – 300
Viscosity (cp)	15 -75	20 – 75
Ash (%)	0.3 – 2.0	0.5 – 2.0

Amino acid compositions of gelatin

Gelatin contains many glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline (4-Hyp) residues. A typical structure is –Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- as shown in Figure 2.6.

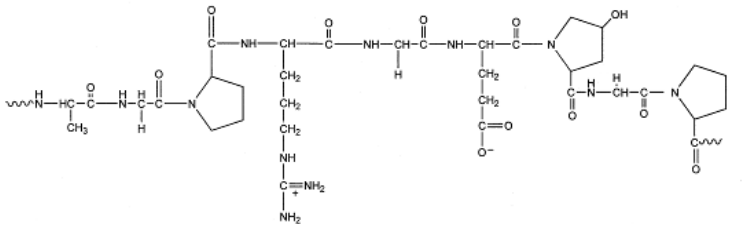

Figure 2.6 Structure of gelatin [18].

Table 2.3 Amino acid composition of gelatin [17].

Amino acid	**wt%**
Arginine	7.8
Glutamic acid	10.0
Histidine	0.8
Hydroxyproline	11.9
Leucine	3.3
Methionine	0.7
Proline	12.4
Theronine	2.1
Valine	2.2
Alanine	8.9
Asperic acid	6.0
Glycine	21.4
Hydroxylysine	1.0
Isoleucine	1.5
Lycine	3.5
Phenylanine	2.4
Serine	3.6
Tyrosine	0.5
Total	100

Properties of gelatin

- Gelatin is colorless or slightly yellow.
- Gelatin is amphoteric. It is neither acidic nor alkali depending on the production process.
- Gelatin swells and absorbs 5 – 10 times its weight of water to form a gel in aqueous solutions at low temperature.
- Gelatin is soluble in hot water, glycerol, and acetic acid, and insoluble in organic solvents.

Applications of gelatin

Gelatin is used in a wide range of industries. In food industry, common examples of foods that contain gelatin are jelly, marshmallows, jams and ice cream. Gelatin is used in food as a gelling, thickener, adhesive, foaming and fining agent. In pharmaceutical industry, gelatin typically constitutes the shells of pharmaceutical capsules in order to make them easier to swallow. Gelatin capsules prevent oxidation and vaporization of the contents. Furthermore, gelatin is used in photographic papers, hair styling-gel and cosmetic industry. The applications of gelatin are shown in Figure 2.7.

Figure 2.7 Applications of gelatin [19].

2.1.1.3 Hydroxyapatite [20, 21]

Hydroxyapatite is a naturally occurring form of calcium apatite with the formula $Ca_{10}(PO_4)_6(OH)_2$. It crystallizes in the hexagonal crystal system. The ratio of Ca:P is 1.67. Pure hydroxyapatite powder is white. Seventy percent of bone is made up of the inorganic mineral hydroxyapatite. It can be used as a filler to replace amputated bone or as a coating to promote bone ingrowth into prosthetic implants. The structure of hydroxyapatite is shown in Figure 2.8.

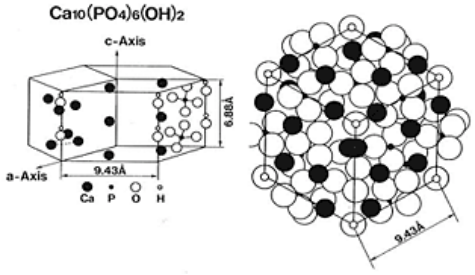

Figure 2.8 Structure of hydroxyapatite [22].

Alternate soaking method is an alternative process of hydroxyapatite formation on/in the substrate. The substrate (such as film, scaffold) is immersed in calcium chloride solution. Then it is removed and immersed in disodium hydrogenphosphate solution. The calcium ions displace free water molecules and repulse each other to give enough space to react with phosphate ions to deposit hydroxyapatite on the surface of the substrate. This is considered as one cycle of alternate soaking.

The reaction of hydroxyapatite formation by alternate soaking process is as follows

$$10CaCl_2 + 6Na_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12NaCl + 8HCl$$

Properties of hydroxyapatite

- Pure hydroxyapatite is white. Naturally occurring apatite has brown, yellow or green colorations**.**
- Hydroxyapatite has a specific gravity of 3.08. It is 5 on the Mohs hardness scale**.**
- Hydroxyapatite is a thermally unstable compound. It is decomposed at temperature about 800 - 1200°C depending on its stoichiometry.

2.1.2. Bone

2.1.2.1 Structure of bone [23]

Bone are rigid organs that form part of the endoskeleton of vertebrates. They function to move, support, and protect the various organs of the body and store minerals.

The structure of bone shown in Figure 2.9 can be grouped into 4 levels.

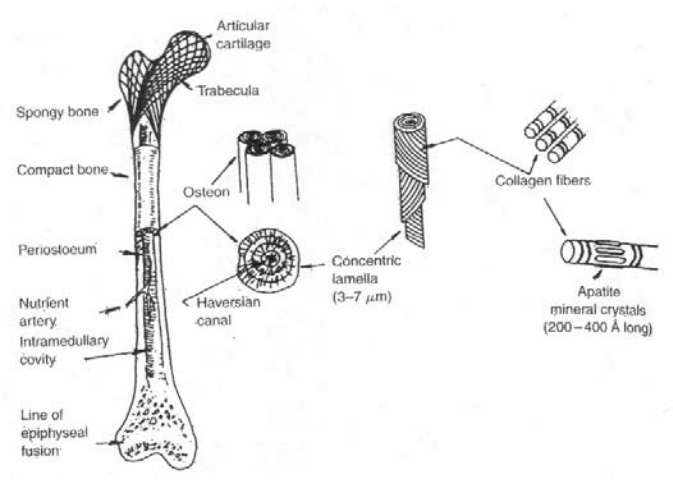

Figure 2.9 Structure of bone [23].

• Molecular level

The smallest unit of structure is the tropocollagen molecule and the associated apatite crystallites. The former is approximately 1.5 x 280 nm, made up of three individual left-handed helical polypeptide chains coiled into a right-handed triple helix. Apatite crystallites have been found to be carbonate-substituted hydroxyapatite. The crystallites appear to be about 4 x 20 x 60 nm^3 in size.

• Ultrastructural level

The collagen and apatite are intimately associated and assembled into a microfibril composite, several of which are then assembled into fibers from approximately 3 to 5 μm thick.

• Microstructural level

These collagen fibers are either randomly arranged (woven bone) or organized into concentric lamellar groups (osteons in the case of human) or linear lamellar groups (plexiform bone in the case of mammals). In addition to the differences in lamellar organization at this level, there are also two different types of architectural structures as shown in Figure 2.10.

- Compact or cortical bone

The hard outer layer of bone is composed of compact and dense bone tissue, due to its minimal gaps and spaces. This tissue gives bones their smooth, white, and solid appearance, and accounts for 80% of the total bone mass of an adult skeleton.

- Cancellous or trabecular bone

Cancellous bone is more porous or spongy. It is composed of a network of rod and plate-like elements that make the overall organ lighter and allowing room for blood vessels and marrow. Cancellous bone accounts for the remaining 20% of total bone mass.

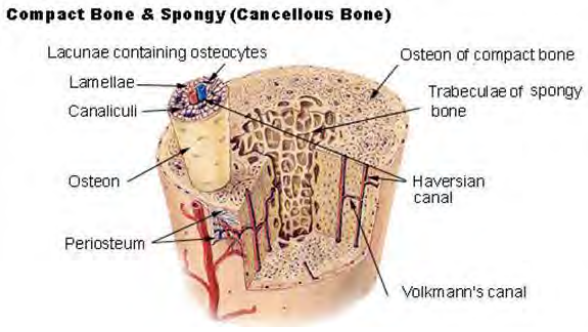

Figure 2.10 Characteristics of compact bone and cancellous bone [1].

• Macrostructural level

Finally, the whole bone constructs of osteons and portions of older, partially destroyed osteons or plexiform bone. The elastic properties of the whole bone results from the hierarchical contribution of each of this levels.

Composition of bone

The composition of bone depends on a large number of factors: the location from which the sample is taken, age, sex, and type of bone tissue, for example, woven, cancellous, cortical. However, a rough estimate for overall composition by volume is one-third apatite, one-third collagen and other organic components, and one-third H_2O. The composition of adult human and bovine cortical bone are given in Table 2.4.

Table 2.4 Composition of adult human and bovine cortical bone [23].

Species	%H_2O	Apatite	%dry weight collagen	Glycosaminoglycan (GAG)
Bovine	9.1	76.4	21.5	Not determined
Human	7.3	67.2	21.2	0.34

2.1.2.2 Mineralization [24]

Bone development can be divided into three stages: proliferation, extracellular matrix maturation and mineralization. For mineralization can be divided into two phases. In the first phase, osteoblasts secrete an organic matrix which is considered to be a preosseous matrix (osteoid). The osteoid consists of type I collagen, proteoglycans, glycoproteins and non-collageneous proteins. During the second phase, mineralization occurs and osteoid is transformed into bone.

Mineralization studies use osteoblastic cells derived from human and rodent bone tissue. In primary cultures, isolated osteoblasts have been shown to synthesize several proteins and enzymes which are known to be localized in bone such as alkaline phosphatase (ALP), osteocalcin and type I collagen. Primary human bone cells are cultured for sufficient lengths of time (about 30 days), nodules is formed by the cells. Nodules are a dense matrix which cells lay down and develop a granular

appearance. These nodules appear to consist of calcium phosphate crystals embedded in matrix. The process is usually associated with the release of vesicle-type structures from the cells and this may initiate mineral deposition.

Culture conditions contribute the ability of cells to differentiate and calcify. The addition of agents such as calcium β-glycerophosphate, glucocorticoids, sodium β-glycerophosphate, calcium hexose monophosphate and dexamethasone are used to promote mineralization of osteoblasts in culture.

2.1.3 **Salt-leaching** [25]

Salt-leaching is a technique that has been used to fabricate silk fibroin scaffolds for tissue engineering applications. The procedures of this method are presented in Figure 2.11.

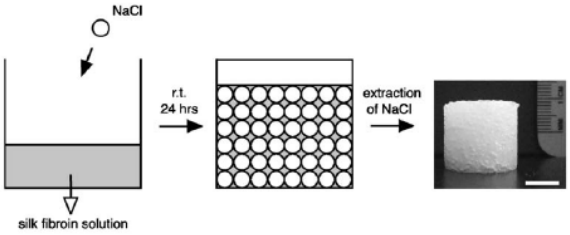

Figure 2.11 Salt-leaching process [25].

Silk fibroin scaffolds were prepared by adding salt crystals with a selected size into silk fibroin aqueous solutions. As salt is composed of of Na^+ and Cl^-. Na^+ is weakly high charge density (kosmotropic) and Cl^- is weakly low charge density (chaotropic). After the salts were added into silk fibroin solution, Na^+ binds water molecules and interacts with oppositely charged residues on the protein surface. Water molecules are easily removed from the proteins as the concentration of salt increases. Silk fibroin consists of 79% of hydrophobic residues. When the salt concentration increases, the hydrophobic interactions between nonpolar residues increase. The hydrophobic interactions induce protein folding, resulting in beta-sheet formation of silk after gelling. The mechanism of salt-leaching process is shown in Figure 2.12.

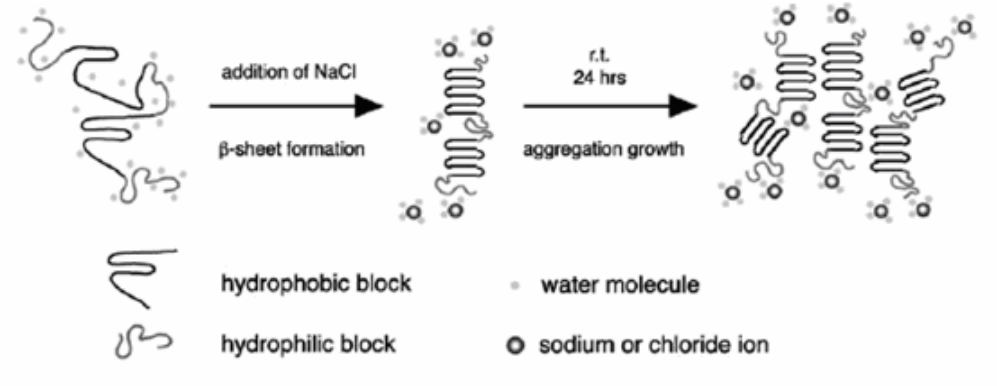

Figure 2.12 Mechanism of salt leaching process [25].

2.1.4 In vitro cell culture

2.1.4.1 Types of cell cultures [26]

Types of cell cultures are classified into two types as follow:

2.1.4.1.1 Primary cell cultures

Primary cell cultures are obtained directly from multiple species including mouse, guinea pig, rat, rabbit, dog, horse, and human. These cells can be kept at the differentiated state for a short period.

Mesenchymal stem cells (MSCs), one type of primary cells, can be isolated from a wide variety of tissues including bone marrow, periosteum, synovium, muscle, adipose tissue, lung, bone, deciduous teeth, dermis, and articular cartilage. Among these, bone marrow is the major source of MSCs. MSCs can be expanded and differentiated into cells of different connective tissue lineages including bone, cartilage, fat, and muscle upon proper stimulation.

These cells also have the potential for a wide range of therapeutic applications through autologous, allogeneic or xenogeneic stem cell transplantation. Bone marrow-derived MSCs have been used to treat a variety of defects and diseases, including critical size segmental bone defects, full thickness cartilage defects, tendon defects, myocardial infarction and even nerve defects.

2.1.4.1.2 Permanent cultures or cell lines cultures

Cell lines cultures have an unlimited proliferation capacity. They are derived from embryos, tumors or transformed cells. Examples of cell lines are L929 mouse skin fibroblast, MC3T3-E1 mouse osteoblast, HeLa, MDCK, etc.

Cells can proliferate and differentiate, both with different limitations, depending on the cell type studied. Numerous publications provide protocols for the isolation of different cell types, their culture conditions, and for the evaluation of the degree of differentiation.

2.1.4.2 Alkaline phosphatase assay [27]

Alkaline phosphatase (ALP) is an enzyme present in almost organism, being particularly high in bone, liver, placenta, intestine and kidney. Alkaline phosphatase is used as a marker of osteoblast phenotype. Alkaline phosphatase has been implicated in the initiation of mineralization. Alkaline phosphatase activity in cell cultures is depended on cell cycle distribution, cell density and length of time in culture. The timing of the phases that accompany differentiation *in vitro* differ depending on the method used, resulting in a lag between the different phases of expression of the individual markers. The production of alkaline phosphatase by human osteoblast cell is detectable from approximately day 4 onwards, rising to a peak from day 10 onwards. A rapid fall in cell proliferation is coincident with a rapid increase in ALP production.

Alkaline phosphatase activity in cell lysate and medium can be determined using a spectrophotometric assay. Alkaline phosphatase catalyses the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol, according to the following reaction:

$$\text{p-Nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-Nitrophenol} + \text{Phosphate}$$

The rate of p-nitrophenol formation is proportional to the catalytic concentration of alkaline phosphatase present in the sample. The absorbance of the solution was measured at 405 nm.

2.1.4.2 O-cresolphythalein assay [28]

Calcium is the mineral present in the largest amount in the body. Approximately 99% of total body calcium is deposited in the skeleton. A higher proportion of non-skeletal calcium is present within cells than in extracellular fluids, and most of this intracellular calcium is bound to proteins in the cell membrane. Intracellular ionized calcium is physiologically active and functions as an intracellular messenger by

binding to or being released from specific intracellular proteins, a process that changes protein conformations and hence its activity or function.

O-cresolphythalein assay, based on the micro-method of calcium determination using O-cresolpthalein complexone dye, involves the reaction of calcium with O-cresolpthalein complexone (O-CPC) to produce a purple complex with an absorbance maximum at 570 nm. The intensity of the color is directly proportional to the concentration of calcium in the sample. This colorimetric assay has been previously used in cell cultures for determination of calcium. The reaction of O-cresolphythalein assay is as follows:

$$Ca^{2+} + \text{O-CPC} \longrightarrow \text{Ca-O-CPC complex (purple color)}$$

2.2 Literature review

In this chapter, the literature reviews are summarized into four parts as follows:

2.2.1 *In vitro* and *in vivo* studies of silk fibroin scaffolds and films.

2.2.2 Preparation and characterization of hydroxyapatite/silk fibroin biomaterials.

2.2.1 *In vitro* and *In vivo* studies of silk fibroin scaffolds and films

In 2005, Ung-Jin Kim *et.al.* [25] studied structural and morphological features of silk fibroin scaffolds prepared from silk fibroin aqueous solutions by salt-leaching method. In this process, the salt promotes water loss from the hydrophobic domains that dominate the fibroin structure. The hydrophobic interactions induce β-sheeted formation. Structural and morphological properties of silk fibroin scaffolds were controlled by the concentration of the silk fibroin solution and the particle size of NaCl used in the process. The scaffolds had highly homogeneous and interconnected pores with pore sizes ranging from 470 to 940 mm. At the same pore size, the compressive strength and modulus of the scaffolds increased with an increase in silk fibroin concentration whereas at the same silk fibroin concentration, the compressive strength and modulus of the scaffolds decreased with increasing in pore size. The degradation by protease was faster with the scaffolds prepared from low silk fibroin concentration. The pore size did not correlate with degradation rate. The scaffolds fully degraded upon exposure to protease during 21 days.

In 2005, Hyeon Joo Kim *et.al.* [29] studied the effect of preparation method in silk fibroin scaffolds for osteogenic responses by human bone marrow stem cells (hMSCs). The three dimensional scaffolds were prepared by either an aqueous process or an organic solvent (hexafluoro-2-propanol, HFIP) process with salt-leaching. The morphology of the aqueous-derived scaffolds showed better pore interconnectivity and rougher surface than the HFIP-derived scaffolds. After 28 days of culture (5×10^5 cells per scaffold), it was found that the rate of cell proliferation, alkaline phosphatase activity and calcium deposition was higher on the water-based silk scaffolds than the HFIP-prepared silk scaffolds. Expression of collagen type I, collagen type II, and osteopontin proteins increased in the water-based silk scaffolds in comparison to the HFIP-derived scaffolds. Histological and immunohistochemical evaluation of silk explants showed the development of bone-like trabeculae with cuboid cells in an extracellular matrix on the water-based silk scaffolds with more organization than on the HFIP-derived material. These data illustrate that macroporous three dimensional aqueous-derived silk fibroin scaffolds provide improved bone-related outcomes in comparison to the HFIP-derived scaffolds.

In 2005, Lorenz Meinel *et.al.* [30] demonstrated the inflammatory response by human bone marrow stem cells (hMSCs) grown on silk films *in vitro* and *in vivo*. The *in vitro* responses of hMSCs on silk were compared with the responses on tissue culture plastic (TCP; negative control), TCP with lipopolysaccharide (LPS) in the cell culture medium (positive control), and collagen films. The cell proliferation was higher on the silk films after 96 h as compared to tissue culture plastic or collagen. On silk both unmodified and RGD-treated, cells formed monolayers in contrast to collagen films where cells formed clusters with a more stratified appearance of the cell layers. The *in vivo* responses on silk and silk-RGD films were compared with the responses on collagen and polylactic acid (PLA) films. For inflammatory responses *in vivo*, films seeded with rat MSCs and cultured for 14 days *in vitro* were implanted intramuscularly in rats. After 6 weeks, the inflammatory *in vivo* response elicited by silk and silk-RGD films was less than that observed on collagen films and far less than to PLA films. Silk explants revealed the presence of circumferentially oriented fibroblasts, few blood vessels, macrophages at the implant-host interface, and the absence of giant cells. These results suggest that silk is biocompatible.

In 2005, Lorenz Meinel *et.al.* [3] evaluated silk fibroin scaffolds for the treatment of bone defects. In *in vitro* tests, human bone marrow stem cells (hMSCs) were seeded on the silk fibroin scaffolds and cultured under osteogenic conditions in bioreactor. Gene expression levels of osteopontin and bone morphogenetic protein 2 (BMP-2) were significantly higher after 3 weeks and decreased after 4 weeks. The alkaline phosphatase activities were increased after 2 weeks. Calcium deposite increased throughout the 5-week period in culture. In vivo responses to tissue-engineered bone, hMSCs-seeded silk fibroin scaffolds, scaffolds alone, and unfilled defects were evaluated in a cranial defect model in nude mice. After 5 weeks, X-ray analysis revealed high levels of mineralization in defects implanted with the tissue-engineered bone. New bone formation was observed on the hMSCs-seeded silk fibroin scaffolds but it did not show on scaffolds alone and unfilled defects. The X-ray results were supported by histological analysis. In μCT analysis, the tissue-engineered bone was the highest new bone formation. The hMSC-seeded silk scaffolds showed the formation of bone more restricted when compared to the tissue-engineered bone group. Little bone formed in defects filled with silk scaffolds alone and in unfilled defects, and the mineralization was mainly restricted to the implant–host interface. These results indicate that silk is feasible for the bone regeneration.

In 2005, Kyoung-Hwa Kim *et.al.* [31] evaluated the biocompatibility of the silk fibroin nanofiber membrane and examined its effect on bone regeneration in a rabbit calvarial model. The silk fibroin nanofiber membrane was fabricated by electrospining. After 7 days of culture MC3T3-E1 osteoblasts, the number of cells attached more than 90% on the surface of membrane and covered all the surface of membrane at 14 days. The alkaline phosphatase activities and osteocalcin production labels were significantly increased at 14 days. In *in vivo* tests, a complete bony union across the defects was observed after 8 weeks. At 12 weeks, the defect had completely healed with new bone. The silk fibroin nanofiber membrane was shown to possess good biocompatibility with enhanced bone regeneration and no evidence of any inflammatory reaction. These results indicate that the silk fibroin membrane should be useful as a tool for guided bone regeneration.

In 2006, Darja Marolt *et.al.* [32] investigated the influences of medium on bone and cartilage tissue constructs in silk fibroin scaffolds. Human bone marrow stromal cells (hMSCs) were seeded on to the specimens at a density 5 x 10^6 cells per scaffold and cultured for 5 weeks in rotating bioreactors using osteogenic, chondrogenic or control medium. Chondrogenic medium yielded constructs with higher DNA content and glycosaminoglycans (GAG) compared to osteogenic and control medium. However, DNA concentrations and GAG concentrations were low (DNA <0.015% wet weight and GAG concentrations <0.05% wet weight) in all groups. The alkaline phosphatase activity was the highest in constructs grown in osteogenic medium as compared to constructs cultured in chondrogenic and control medium. Osteogenesis was further confirmed by high amount of calcium in constructs grown in osteogenic medium. Compressive modulus were twice as high in the cell-based scaffolds when compared to unseeded scaffolds. However, compressive modulus were relatively low in all groups. The collagen type I and type II were found on the scaffolds cultured in chondrogenic medium. For alcian blue staining, GAG was minimally found in chondrogenic and osteogenic medium but it was not found in control medium. The von Kossa assay and μCT analyse showed the presence of mineralized structures on the scaffolds cultured in osteogenic medium whereas no mineralized structures were detected in the scaffolds cultured in chondrogenic medium and control medium.

In 2007, Sandra Hoffmann *et.al.* [33] reported the effect of pore size and seeding process on osteogenic differentiation of human bone marrow stem cells (hMSCs) in silk fibroin scaffolds. Silk fibroin scaffolds were prepared by organic solvent (hexafluoro-2-propanol, HFIP) process using salt-leaching method. The silk fibroin scaffolds with small pores (112 – 224 μm), large pores (400 – 500 μm) and with pore diameters of 112 – 224 μm on one side and 400 – 500 μm on the other side of the scaffold were fabricated. Dynamic seeding and static seeding were evaluated with respect to cell attachment and proliferation capacity. Static seeding resulted in higher amounts of DNA per mg wet weight than the dynamic seeding method whereas no difference was observed among the different pore sizes. Dynamic seeding restricted cell deposition to viable cells whereas static seeding led to the deposition of both dead and viable cells. Both seeding strategies were equivalent in terms of cell viability and cell activity. After dynamic seeding, cells had a more globular cell

structure and attached closely to the silk fibroin lattice whereas after static seeding, cells were located in the void spaces. After 3 and 5 weeks of cultivated in osteogenic medium, alkaline phosphatase activity and calcium deposition increased when compared to control medium. Images taken with micro-computed tomography of hMSCs-seeded mixed pore scaffolds grown under osteogenic conditions for 3 weeks demonstrated the formation of bone-like tissue whereas in control medium no mineralization was found.

In 2007, Panya Wongpanit *et.al.* [34] improved dimensional stability of silk fibroin sponge by incorporating chitin whiskers as nanofibers. The composite sponges at chitin whiskers to silk fibroin weight ratio (C/S ratio) of 0, 1/8, 2/8, or 4/8 were fabricated using a freeze-drying technique. TEM micrograph indicated that the dispersion of chitin whiskers embedded in the silk fibroin matrix was found to be homogenous. The average length and width of chitin whiskers was about 427 and 43 nm, respectively. The percent shrinkage of scaffolds after methanol treatment decreased with an increasing of the whisker content and finally reached the plateau at C/S ratio of 2/8. After treated methanol, all C/S ratios sponges and pure silk fibroin sponges showed the β-sheet structure. The decomposition temperature of pure silk fibroin appeared to be at the sharper endothermic peak of 287 °C and the sharper endothermic peak of 304 °C. The C/S ratio of 1/8 induced the sharp peak from 304 to 298 °C. The further increase in the amount of chitin whiskers resulted in the absence of the sharp endothermic peak of the decomposition temperature. The chitin whiskers embedded into silk fibroin sponges enhanced the compression strength. The morphology showed that all samples were interconnected pore networks with an average pore size of 150 μm. L929 cells (1×10^5 cells per scaffold) were seeded onto the pure silk fibroin sponges and C/S ratio of 4/8 sponges. The result indicated that there was no significant difference between both types of at 6 h of cultivation. After 24 h of cultivation, the cell spreading on chitin whisker/silk fibroin sponges was twice compared to that on the pure silk fibroin sponges. SEM micrograph revealed that the cells exhibited different shapes, including flattening, typical elongated fibroblast cell shapes and spherical shapes at 6 h of cultivation. After 24 h of cultivation, the number of cells with extended filopodia was observed to be relatively higher compared to at 6 h. An observed number of flattened cells on the chitin whisker/silk fibroin sponges

were higher than that on the pure silk fibroin sponges. The results showed that silk fibroin sponges both with and without chitin whiskers were cytocompatible. These results might indicate the potential utility of this nanocomposite system for further exploration as a scaffolding material.

2.2.2 Preparation and characterization of hydroxyapatite/silk fibroin biomaterials

In 1999, Tsutomu Furuzono *et.al.* [35] developed the alternate soaking process to silk fabric to prepare a composite of silk fabric and apatite. Silk fabric 1.5 cm in diameter was immersed in calcium solution (200 mM aqueous calcium chloride solution buffered with tris –aminomethane and HCl) for 1 h followed by 120 mM disodium hydrogenphosphate for 1 h. Soaking was alternated for about 30 cycles. Hydroxyapatite deposition increased with soaking repetitions. SEM showed that hydroxyapatite deposited after 21 or more repeated soaking was over 20 μm thick. In the XRD patterns of hydroxyapatite-silk fabric composites, a broad peak around 20° (2θ) was attributed to the silk II form in which crystalline regions had an identical orientation. Reflection peak in XRD pattern became sharper with increasing soaking repetitions, indicating a higher crystallinity. FTIR indicated the existence of carbonate, HPO_4^{2-} and Na^+ ions in addition to constituent ions of hydroxyapatite. The x-ray photoelectron spectroscopy (XPS) examined spectrum for undeposited silk showed C, N and O. Peaks of Ca, P, O, Na, N appeared in the XPS spectrum of the hydroxyapatite-silk fabric composites. The absence of the N peak indicated that the silk fiber surface after three alternate soaking was fully covered. These result illustrated that hydroxyapatite deposited on silk fabric using alternate hydroxyapatite forming system might be potentially bioactive similar to natural bone tissue.

In 2007, Rikako Kino *et.al.* [36] studied the deposition of hydroxyapatite crystals from a simulated body fluid (SBF) on silk fibroin films prepared with and without calcium ions. SBF is the solution containing $CaCl_2$, $NaHCO_3$, KCl, $K_2HO_4{\cdot}3H_2O$, $MgCl_2$, $CaCl_2$, and Na_2SO_4. The stability of β-sheet structure was improved by the addition of calcium ions and the subsequent MeOH treatment. Silk fibroin films containing >5 wt% calcium chloride were coated with hydroxyapatite

crystals after soaking in 1.5-times SBF for 6 h, while films with calcium chloride contents lower than 3 wt% were not mineralized under these conditions. The intensity of hydroxyapatite diffractions increased with soaking times, the thickness of hydroxyapatite deposited layer could be regulated by soaking times. At 6 h after soaking silk fibroin films containing 5 wt% calcium chloride in 1.5-times SBF, the dots with 200 – 300 nm in diameter attributed to the hydroxyapatite crystals first appeared. Soaking in 1.5-times SBF for 12 h promoted the formation of irregularly spherical aggregates of hydroxyapatite evenly covering the silk fibroin films surface. The Ca/P ratio was 1.15 for silk fibroin films containing 5 wt% calcium chloride at 6 h, 1.48 at 12 h and 1.65 at 24 h, approaching the theoretical value of 1.67 for hydroxyapatite. These result suggested that the simple addition of calcium ions to regenerated silk fibroin may provide a useful method for producing hydroxyapatite-mineralized materials for bone repair.

In 2007, Toshimitsu Tanaka *et.al.* [37] evaluated the efficiency of a nano-scaled hydroxyapatite/silk fibroin (nano-HAp/SF) sheet, as bone-regeneration scaffolds using rat bone marrow mesenchymal cells (MMCs). The nano-HAp/SF sheets were prepared by mixture of methacryloxypropyl trimethoxysilane (MPTS), ammonium peroxodisulfate, pentaethylene glycol dodecyl ether and silk fibroin fibers in tube. The polymerization was conducted at 50 °C for 50 min and then washed with ethanol and dried in a vacuum for 1 h at 60 °C. Poly(MPTS)-grafted SF was soaked in the solution of nano-HAp particles for 1 h at room temperature. Then it was heated at 120 °C for 2 h in a vacuum. The experiments demonstrated that the nano-HAp/SF sheets showed good initial cell attachment and supported cell proliferation. After 14 days of culturing under osteogenic conditions, the alkaline phosphatase (ALP) activity and bone-specific osteocalcin secretion of the cells on nano-HAp/SF sheets were higher than silk fibroin sheets. The nano-HAp particles support the good adhesion of the cell to the nano-HAp/SF sheets because the roughness of sheet surface are increased. Otherwise cell adhesion molecules such as fibronectin in serum or the molecules produced by the cells could more easily support cellular adhesion and proliferation since many proteins are actively adsorbed on HAp surfaces. These results indicated that the HAp support the osteogenic diffentiation of MMCs. Therefore, the nano-HAp/SF sheet is an effective biomaterial that is applicable in bone reconstruction surgery.

In 2007, Rikako Kino *et.al.* [38] used silk fibroin (SF) and hydroxyapatite (HAp)-deposited silk fibroin film to prepare multilayered films by alternating lamination. HAp-deposited SF films were prepared from SF solution containing 5 wt% $CaCl_2$ by air drying, treated with methanol vapor and then soaked in simulated body fluid. The multilayered HAp/SF films had HAp layers with approximate thickness of 3 – 5 μm and SF layers with thickness of 40 – 70 μm. The multilayered HAp/SF films were prepared by alternating lamination at 130 °C for 4 min to achieve a higher bonding strength and a higher β-sheet content. The comparison of mechanical strength between the multilayered HAp/SF films and multilayered SF films indicated that HAp layered increased the film's mechanical strength. The biocompatibility and osteoconductivity of HAp-deposited SF films was analyzed by culturing of mice osteoblasts (MC3T3-E1). The cell cultivation indicated that HAp-deposited SF films and SF films show similar degrees of cell adhesion and alkaline phosphatase activities.

In 2008, Hyeon Joo Kim *et.al.* [39] investigated of bone-like mineral hydroxyapatite into silk fibroin scaffolds for bone tissue engineering. Porous silk fibroin/polyaspartic acid scaffolds were prepared using a salt-leaching method (particle size of NaCl : 850–1000 μm). The blend ratios of silk fibroin/polyaspartic acid were 100/0, 95/5, 90/10 and 80/20 (w/w). The scaffolds were soaked in 200 mM $CaCl_2$ solution for 20 minutes and followed by 120 mM Na_2HPO_4 solution for 20 minutes in order to grow hydroxyapatite on scaffolds. The soaking cycles were repeated 3, 5 and 7 times. The pore sizes of silk fibroin scaffolds were 750±20 μm. The scaffold pores were highly interconnected. The x-ray photoelectron spectroscopy results showed that the amount of hydroxyapatite in the scaffolds increased with increase in polyaspartic acid content and mineralization cycles. hMSCs ($5x10^6$ cells/scaffold) were seeded on all four groups of silk fibroin/polyaspartic acid scaffolds with five cycles of mineralization and maintained with or without BMP-2 in osteogenic medium for 6 weeks. The cell numbers on mineralized 80/20 scaffolds were slightly higher than those on the other mineralized scaffolds. Cell proliferation was not affected by BMP-2 throughout the scaffolds. In the absence of BMP-2, ALP activity and calcium deposition were similar in all groups of scaffolds. The mineralized 80/20 scaffolds showed the highest of ALP activity. Calcium deposition presented an increase content of polyaspartic acid in all mineralized silk scaffolds

with BMP-2. Hematoxylin and eosin indicated that the polyaspartic acid content was increased the mineral deposition increased and cells were less uniformly distributed in the scaffolds. Von Kossa staining supported calcification on the surface of scaffolds. Theses results suggest increased osteoconductive outcomes with an increase in initial content of hydroxyapatite and BMP-2 in the silk fibroin porous scaffolds. The premineralization of these highly porous silk fibroin protein scaffolds provided enhanced outcomes for the bone tissue engineering.

In 2008, Feng Lin *et.al.* [40] investigated the deposition behavior of hydroxyapatite crystal on the silk fibroin scaffolds by soaking in the simulated body fluid for different times. Porous 3D silk fibroin scaffolds were prepared from 2.0% (w/v) silk fibroin solution with the addition of 0.5% (v/v) methanol and 0.2% (v/v) glutaraldehyde by a freeze-drying method. The scaffolds were then soaked in the simulated body fluid for 12 h, 1, 3, 5 and 7 days. The simulated body fluid were prepared according to the ion concentration of human blood plasma. The XRD and FTIR results showed that the silk fibroin scaffolds were in β-sheet structure. The XRD and AAS data indicated that the silk fibroin scaffolds could induce the continuous growth and enrichment of hydroxyapatite crystals onto the scaffolds with the increase of soaking time. The morphology of silk fibroin scaffolds soaked for less than 3 days showed the smooth porous surface similar to the scaffolds without soaking. In contrast, the porous surface of scaffolds soaked for 5 and 7 days became rough. All the scaffolds were more than 80% porosity and were excellent water-absorptivity (760-870%). The mechanical properties of scaffolds increased first with the hydroxyapatite deposition within 3 days of soaking, then it declined. The human osteosarcoma cell line MG-63 (1×10^5 cells per scaffold) cultivation indicated that the cells could adhere well on the scaffolds. The attachment for each sample was more than 70% when the cells were cultivated for 4 h. The number of cells increased when cultivation time was 24 h. No significant difference on the attachment was observed between the different scaffolds when the cells were cultivated for 4 h. However, a gradual increase was found with the extension of soaking time when the cells were cultivated for 24 h. This result suggested that the hydroxyapatite deposition onto the scaffolds promoted the cell biocompatibility. The scaffolds might be a potential candidate in the bone engineering.

In 2009, Chunling Du *et.al.* [41] investigated the structure and properties of silk fibroin/hydroxyapatite composite films which may provide some information for the biomimetic processing of such kind of composite materials. The silk fibroin/ hydroxyapatite composite films were fabricated by co-precipitation, where the theoretical hydroxyapatite contents were 2, 6, 12, 21, and 31% (w/w). 0.1 mol/l $Ca(NO_3)_2$ solution was slowly dropped into 2.2% (w/v) silk fibroin solution with addition of 0.5% (v/v) glycerin followed by adding 0.1 mol/l Na_2HPO_4 solution. During the dropping process, the ultrasonication was used for the dispersion of inorganic components into silk fibroin. The mixtures were casted onto the disks (diameter 90 mm) and dried at 37 °C overnight. The morphology of composite films were smooth and transparent with the uniform distribution of hydroxyapatite into the composites when the final hydroxyapatite content was lower than 21% (w/w). The AFM optical microscopy images indicated that the roughness of films increased with the increase of inorganic component. XRD data showed that the silk fibroin in the composite was β-sheet crystalline structure which was induced not only by the addition of glycerin also by the hydroxyapatite crystal growth during the composite fabrication. The hydroxyapatite crystals were the anisotropic growth with high extent of lattice imperfection and the preferential orientation along c-axis, probably promoted by the silk fibroin. The decomposition peak of silk fibroin films was at 289 °C and 308 °C. This suggested that a certain content of amorphous structure was remained in the silk fibroin film. The shoulder at 289 °C disappeared and another shoulder at higher temperature side appeared alternatively with the increase of inorganic components. So that, the molecular orientation and the crystallinity of silk fibroin were improved by the hydroxyapatite formation during the film fabrication. The mechanical testing results indicated that both break strain and stress were declined with the increase of hydroxyapatite content in the composites, presumably due to the original brittleness of hydroxyapatite compound.

CHAPTER III

EXPERIMENTAL WORK

3.1 Materials

3.1.1 *Bombyx mori* cocoon (Nangnoi Srisaket1 from The Queen Sirikit Institute of Sericulture, Nakhonratchasima province, Thailand)

3.1.2 Type A gelatin powder (pI 9, Nitta Gelatin Inc., Japan)

3.1.3 Sodium carbonate (Na_2CO_3, Ajax Finechem, Australia)

3.1.4 Lithuim bromide (LiBr, Sigma-Aldrich, Germany)

3.1.5 Mined salt (particle size 600-710μm, Thai refined salt Co.Ltd., Nakhonratchasima, Thailand)

3.1.6 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Nacalai Tesque, Inc., Japan)

3.1.7 N-hydroxysuccinimide (NHS, Nacalai Tesque, Inc., Japan)

3.1.8 Calcium chloride ($CaCl_2$, Ajax Finechem, Australia)

3.1.9 Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$, Sigma-Aldrich, Germany)

3.1.10 Ethanol (99.7-100%, VWR International Ltd., UK)

3.1.11 Alpha-modified eagle powder medium (α-MEM, Hyclone, USA)

3.1.12 Phosphate buffer saline without calcium, and magnesium (PBS, Nissui Pharmaceutical Co. Ltd, USA)

3.1.13 Trypsin/EDTA (0.25% trypsin in EDTA·4Na, Hyclone, USA)

3.1.14 Fetal bovine serum (FBS, Hyclone, USA)

3.1.15 Penicillin-Streptomycin solution (10,000 units/ml, Hyclone, USA)

3.1.16 Sodium hydrogen carbonate (Fluka, Germany)

3.1.17 Glutaraldehyde solution (50% GTA, Fluka, Germany)

3.1.18 Hexamethyldisilazane (HMDS, Fluka, Germany)

3.1.19 Trypan blue solution (0.4%, Sigma-Aldrich, Germany)

3.1.20 Dexamethasone (Sigma-Aldrich, Germany)

3.1.21 L-Ascorbic acid (Sigma-Aldrich, Germany)

3.1.22 β-glycerophosphate (Fluka, Germany)

3.1.23 P-Nitrophenol standard solution (10mM, Sigma-Aldrich, Germany)

3.1.24 P-Nitrophenyl phosphate liquid substrate (Sigma-Aldrich, Germany)

3.1.25 Sodium hydroxide (NaOH, Analar, England)

3.1.26 Wistar rats (3-week-old, Female, order from Animal research center, Mahidol University)

3.2 Equipments

3.2.1 -50°C freezer (Heto, PowerDry LL3000, USA)

3.2.2 Lyophilizer (Heto, PowerDry LL3000, USA)

3.2.3 Vacuum drying oven and pump (VD23, Binder, Germany)

3.2.4 Fine coat (JFC-1100E, JEOL Ltd., Japan)

3.2.5 Scanning Electron Microscopy (JSM-5410LV, JEOL Ltd., Japan)

3.2.6 Universal Testing Machine (Instron, No. 5567, USA)

3.2.7 Thermogravimetic analyzer (SDT Q600, USA)

3.2.8 Laminar Flow (HWS Series 254473, Australia)

3.2.9 CO_2 incubator (Series II 3110 Water Jacketed Incubator, Thermo Forma, USA)

3.2.10 UV-VIS spectrophotometer (Thermo Spectronic, Genesys 10UV scanning, USA)

3.2.11 Fluorescence microplate reader (Perkin elmer, 1420 multilabel counter, USA)

3.2.12 24-well and 48-well polystyrene tissue culture plates (Corning, Germany)

3.2.13 T75 tissue culture flask (Cellstar, USA)

3.2.14 Micropipette (Pipetman P20, P200, P1000 and P5000, USA)

3.2.15 Hemacytometer (Counting chamber, Boeco, Germany)

3.3 Experimental procedures

This experimental procedures can be divided into three main steps: preparation of Thai silk fibroin scaffolds, gelatin conjugation and hydroxyapatite deposition on Thai silk fibroin-based scaffolds, and characterization of the physical and biological properties of Thai silk fibroin-based scaffolds. All experimental procedures are summarized in Figure 3.1.

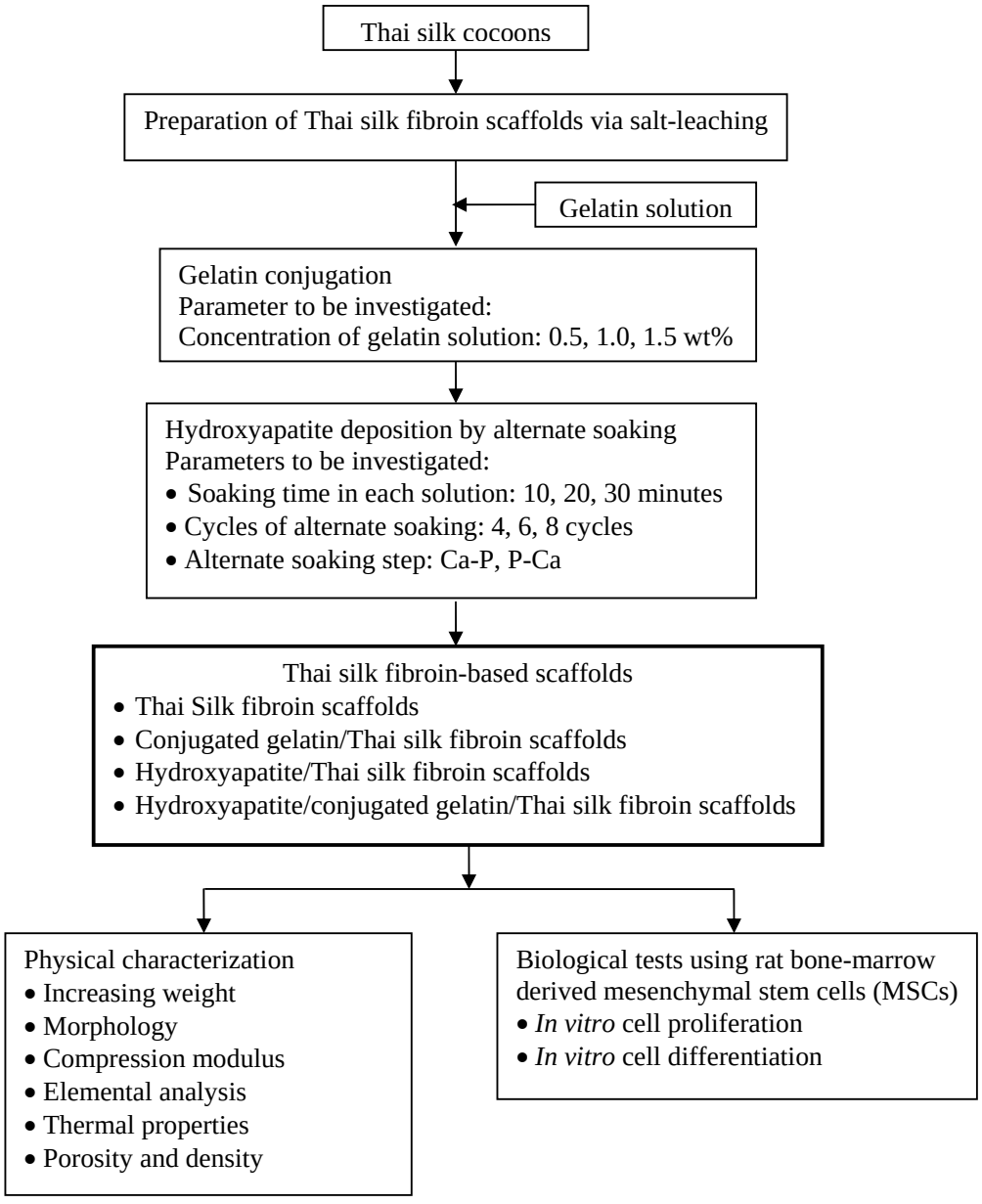

Figure 3.1 Summary of experimental procedures.

3.3.1 Preparation of Thai silk fibroin and gelatin solutions

Thai silk fibroin solution was prepared as described by Kim *et.al.* [25]. Cocoons of *Bombyx mori* were boiled for 15 min in an aqueous solution of 0.02 M Na_2CO_3. This process was repeated two times to remove sericin. The Thai silk fibroin fibers were dried at room temperature overnight. After drying, the degummed Thai silk fibroin fibers ware dissolved in 9.3 M LiBr solutions at 60^{o}C for 4 h to form 25wt% solution. The solution was dialyzed against deionized water at room temperature for 2 days (seamless cellulose tubing: molecular weight cut off 12000-16000) till the conductivity of dialyzed water was the same as that of deionized water. After dialysis, 6.5wt% of aqueous Thai silk fibroin solution, determined by weighting the remaining solid after drying, was obtained.

To prepare gelatin solution, type A gelatin was swollen in deionized water at room temperature for 30 min and then dissolved at 40°C under agitation for 60 min to obtain 0.5wt%, 1.0wt% and 1.5 wt% solutions.

3.3.2 Preparation of Thai silk fibroin and Thai silk fibroin-based scaffolds

3.3.2.1 Preparation of Thai silk fibroin scaffolds

After dialysis, 3ml of 6.5wt% Thai silk fibroin solution was added in a cylindrical container 9 g of mined salt (particle size: 600-710µm) was slowly added in Thai silk fibroin solution. The container was covered and left at room temperature for 24 h to allow the gelation of silk fibroin solution. Then, the container was immersed in water to leach out salt for 2 h. The scaffold was taken out from the container and immerse in water under stirring for 4 h to ensure complete removal of salt. The washing water was changed every 30 min. After that, the scaffold was air-dried overnight.

3.3.2.2 Preparation of conjugated gelatin/Thai silk fibroin scaffolds

The silk fibroin scaffold was punched into 11 mm in diameter, 2 mm in height and immersed in gelatin solution at each desired concentration under vacuum for 2 h to allow gelatin coating on silk fibroin scaffolds. The ratio of gelatin solution to weight of scaffold was 1 ml : 1.5 mg. After that, it was frozen at -50°C overnight prior lyophilized at -55°C for 24 h. To conjugate gelatin, the freeze-dried scaffolds were treated by dehydrothermal (DHT) treatment at 140°C for 48 h in a vacuum oven. After that, the scaffolds were treated with carbodiimide solution (14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5.5 mM N-hydroxysuccinimide (NHS)) at room temperature for 2 h. Conjugated scaffolds were immersed deionized water for 30 min to remove excess NHS and EDC. The deionized water was changed every 10 min. This process was repeated three times. The obtained scaffolds were then dried at room temperature.

3.3.2.3 Preparation of hydroxyapatite/Thai silk fibroin and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds

Hydroxyapatite was deposited into the scaffolds by an alternate soaking process using calcium chloride ($CaCl_2$) and disodium hydrogenphosphate (Na_2HPO_4) solutions. The ratio of calcium and phosphate solutions to the weight of scaffold was 1 ml : 1.5 mg. Thai silk fibroin and conjugated gelatin/Thai silk fibroin scaffolds were first immersed in 0.2 M $CaCl_2$ at room temperature under vacuum for 30 min. After that, the scaffolds were removed from $CaCl_2$ solution and rinsed with deionized water. The scaffolds was then immersed in 0.12 M Na_2HPO_4 at room temperature under vacuum for 30 min. After removing the scaffold from Na_2HPO_4 solution, it was rinsed with deionized water again. This was considered as one cycle of alternate soaking. The alternate soaking was performed for 4, 6 and 8 cycles. Fresh $CaCl_2$ and Na_2HPO_4 solutions were used for each soaking cycle. After desired cycles of soaking process, the hydroxyapatite/Thai silk fibroin and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds were air-dried at room temperature.

To study the effects of alternate soaking step, the immersing solution used to begin the alternate soaking was compared as follows.

Process 1 : First immersing in $CaCl_2$ solution, following by Na_2HPO_4 solution.

Process 2 : First immersing in Na_2HPO_4 solution, following by $CaCl_2$ solution.

3.3.3 Characterization of scaffolds

3.3.3.1 Physical characterization

3.3.3.1.1 Increasing weight of scaffolds

The weight percentage of hydroxyapatite deposited was determined from the weight increased after alternate soaking as follows.

$$\%HA = \frac{W_2 - W_1}{W_1} \times 100$$

Where W_2 and W_1 is the weights of dried scaffold after and before alternate soaking, respectively. The reported values were the mean±standard deviation (n=5).

3.3.3.1.2 Morphology

The morphology of scaffolds was investigated by scanning electron microscopy (SEM). In order to observe the inner structure of scaffolds, the scaffolds were cut vertically with razor blades. The cut scaffolds were placed on the copper mount and coated with gold prior to SEM observation. Pore size was obtained using SemAfore software (n=50).

3.3.3.1.3 Compressive modulus

The compression tests were performed on all scaffolds using a universal testing machine (Instron, No. 5567) at the constant compression rate of 0.5 mm/min. The compressive modulus of the scaffolds (dimension: d = 11 mm, h = 3mm) was

determined from the slope of the compressive stress-stain curves during the strain range of 5% - 30%. The reported values were the mean±standard deviation (n=5).

3.3.3.1.4 Elemental analysis

Energy dispersive x-ray spectrometer was used to evaluate element component in scaffolds. The sample was mounted on a stub by carbon tape. It was analyzed using a scanning electron microscopy equipped with an energy dispersive x-ray spectroscopy (SEM-EDX) using an acceleration voltage of 10 kV. The surface of sample was scanned for micro-sized particles. The diameter of scanning probe was 5 μm. A semi-quantitative elemental analysis was then performed on these samples using EDX.

3.3.3.1.5 Thermal properties

Thermogravimetic analysis was used to evaluate the ratio of organic to inorganic components contained in the scaffolds as well as the degradation temperature of the scaffolds. All experiments were performed in the scanning mode from 35 to 1000°C at the heating rate of 20°C/min under oxygen atmosphere. The initial weight of sample was about 5 mg.

3.3.3.1.6 Porosity and density

The porosity of the scaffolds was measured by liquid displacement [25]. Ethanol was used as the displacement liquid as it could permeate through the scaffolds. The volume of 1 ml ethanol were recorded as V_1. The scaffolds (dry weight, W_d) were immersed in the ethanol under vacuum for 5 min and the volume of scaffolds in ethanol were recorded as V_2. The ethanol impregnated-scaffolds were then taken out and the volume of residue ethanol was recorded as V_3. The total volume of the scaffold (V) was

$$V = (V_2 – V_1) + (V_1 – V_3) = V_2 – V_3$$

$V_2 - V_1$ is the volume of the scaffold and $V_1 - V_3$ is the volume of ethanol within the scaffold. The porosity of the scaffold (ε) was obtained by

$$\varepsilon(\%) = \frac{(V_1 - V_3)}{(V_2 - V_3)} \times 100$$

The density of the scaffold (ρ) was calculated from the following equation.

$$\rho(\mathrm{mg/mm^3}) = \frac{V_d}{(V_2 - V_3)}$$

3.3.3.2 Biological characterization

3.3.3.2.1 MSCs isolation and culture

MSCs were isolated and cultivated according to the methods reported by Takahashi [2]. MSCs derived from the bone shaft of femurs of 3 week-old wistar rats. Both ends of rat femurs were cut away from the epiphysis and the bone marrow was flushed out by a 18-gauge needle with 1 ml of modified eagle medium (α-MEM) supplemented with 15% fetal bovine serum. The cell suspension was placed into tissue culture plates containing α-MEM medium with 15% fetal bovine serum at 37ºC in 5% CO_2 incubator. The medium was changed on the 4^{th} day of culture and every 3 days thereafter. When the cells proliferated became subconfluent, around 7-10 days, the cells were detached with 0.25wt% of trypsin and divided into a new plate. The cells of the second and third passage at subconfluence were used in further study.

3.3.3.2.2 *In vitro* cell proliferation tests

The scaffolds were placed in a 48-well culture plate and sterilized by ethylene oxide gas. Before cell seeding, 350 μl of α-MEM medium with 15% FBS was added into each well of the 48-well plate. 5×10^5 of MSCs were seeded into each scaffold and incubated on orbital shaker at 200 rpm, 37°C in 5%CO_2 incubator for 6 h. After that, the scaffolds were removed to 6-well culture plate and cultured for 6 h, 3 and 5 days. The culture medium was changed every three days. After cultured for a desired period, the cells were then quantified by DNA assay [42]. The samples were washed with PBS and lysed in SDS lysis buffer at 37°C overnight. After that, 100 μl of each

sample (cell lysate) was pipetted into 96-well black plate. Hoechst solution was added into the cell lysate in order to bind with DNA in cell lysate. The fluorescent intensity of the solution was measured in a fluorescence spectrometer at the excitation and emission wavelengths of 355 and 460 nm, respectively. All data were expressed as mean±standard deviation (n = 3).

3.3.3.2.3 *In vitro* cell differentiation tests

Osteogenic differentiation of MSCs cultured on the scaffolds under osteogenic induction was assessed. After the scaffolds were sterilized by ethylene oxide gas, MSCs were seeded on the scaffolds as described previously ($1x10^6$ cells/scaffold) and cultured in α-MEM medium with 15% FBS at 37°C, 5% CO_2. After one day of seeding, the medium was changed into osteogenic medium (α-MEM, 10% FBS, 10 mM β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10 nM dexamethasone). α-MEM supplemented with 10% FBS was used as control medium of osteogenic differentiation. The culture medium was changed every 2 days. After 7, 14, 21 and 28 days cultured in osteogenic medium and control medium, osteogenic differentiation markers of the cell lysate, including alkaline phosphatase (ALP) activity and calcium content, were determined. The cell lysate was prepared by washing the sample with PBS and lysing the sample in SDS buffer at 37°C overnight.

For ALP assay [2], the dilution of p-nitrophenol solution (standard solution) was used as a standard curve. 20 μl of cell lysate was transfered into a 96-well plate. After that, 100 μl of p-nitrophenyl phosphate liquid substrate was added into each cell lysate sample and standard solution. After 15 min of incubation at 37°C, 0.02 N NaOH was added into each well to stop reaction. ALP in cell lysate catalyses the hydrolysis of p-nitrophenyl phosphate, liberating p-nitrophenol and phosphate. The absorbance of the solution was measured at 405 nm.

For calcium assay [43], 1 M HCl was added into cell lysate samples with equal volume and the sample was incubated at 4°C for 4 h. HCl helps to release calcium which bound to cell membrane in the cell lysate. 1 ml of 0.88 M ethanolamine buffer was added into a 48-well plate followed by 10 μl of sample and 100 μl of 0.63 M O-cresolphythalein complex substrate. After that, 300 μl of the mixed solution was transfered into a 96-well plate. The absorbance of the solution

was measured at 570 nm. The standard curve was prepared by using the calcium standard solution (50 mg/ml $CaCO_3$ in 1M HCl) instead of the sample solution. It was noted that, for the case of scaffold containing hydroxyapatite, the original amount of calcium in each scaffold was determined and used to subtract from the amount of calcium of that scaffold obtained after cell culture.

At each time interval, the number of cells were also determined by DNA assay and used to normalize the ALP activities and calcium contents. All data were expressed as mean±standard deviation (n = 3).

3.3.3.2.4 Migration and morphology of cultured MSCs

Cultured cells in each scaffold were fixed with 2.5% glutaraldehyde solution in PBS for 1 h. Scaffolds were then serially dehydrated by a series of ethanol, which were 30%, 50%, 70%, 80%, 90%, 95% and 100%, for 5 min at each concentration. 200 μl of hexamethyldisilazane (HMDS) was added to dry the dehydrated scaffolds at room temperature. Dried scaffolds were cross-sectional cut. The cell migration and morphology of cultured MSCs was observed by SEM.

3.3.4 Statistical analysis

Significant levels of all results were determined by an independent two-sample t-test. All statistical calculations were performed on the Minitab system for Windows (version 14, USA). P-values of <0.05 was significantly considered.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Effects of gelatin concentration on conjugated gelatin/Thai silk fibroin scaffolds

4.1.1 Morphology of scaffolds

As gelatin was coated and conjugated onto the pore surface of Thai silk fibroin scaffolds, it was essential to determine the amount of conjugated gelatin onto the scaffolds from increasing weight after dehydrothermal and EDC conjugating (crosslinking) process. The increasing weight of gelatin compared to pure Thai silk fibroin scaffolds was determined after three steps as follows (see Figure 5.1).

(1) after gelatin coating (before DHT crosslinking)

(2) after DHT crosslinking

(3) after EDC crosslinking

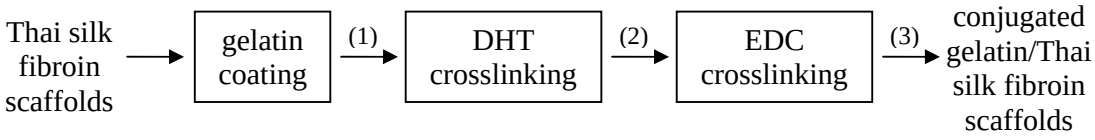

Figure 4.1 Steps of gelatin conjugating.

All types of conjugated gelatin/Thai silk fibroin scaffolds prepared were listed in Table 4.1.

Table 4.1 List of conjugated gelatin/Thai silk fibroin scaffolds prepared in this work.

Scaffold type	Concentration of gelatin	Notation
Conjugated gelatin/Thai silk fibroin scaffolds	0wt%	SF
	0.5wt%	0.5%CGSF
	1.0wt%	1.0%CGSF
	1.5wt%	1.5%CGSF

Figure 4.2 presented increasing weight of gelatin incorporated in Thai silk fibroin scaffolds using three different concentrations of gelatin solution. The results showed that, at each constant concentration of gelatin used, the increasing weight of scaffolds after the steps of dehydrothermal treatment was slightly decreased from that before dehydrothermal treatment. This could be the result of thermal dehydration occurred during DHT crosslinking which theoretically generate chemical bonding between adjacent amino and carboxyl groups of protein molecules. After DHT crosslinking, the scaffolds were further conjugated (crosslinked) with EDC/NHS solution. The increasing weight of scaffolds was found to be slightly decreased due to the dissolution of uncrosslinked part of gelatin while washing scaffolds with water.

Comparing the effects of gelatin concentration used, it was noticed that as increasing gelatin concentration from 0.5wt% to 1.5wt%, the amount of conjugated gelatin onto the scaffolds was increased. This indicated that high concentration of gelatin solution used could allow more gelatin contents to conjugate onto Thai silk fibroin scaffolds.

The morphology of the Thai silk fibroin and conjugated gelatin/Thai silk fibroin scaffolds prepared from gelatin solution with different concentration was shown in Figure 4.3. Thai silk fibroin scaffolds presented an interconnected porous network with smooth porous surface (Figure 4.3a-4.3b). The pore size of scaffold structure corresponded to the size of salt crystals used. Considering the effects of gelatin concentration, the morphology of conjugated gelatin/Thai silk fibroin scaffolds was shown to depend upon the concentration of gelatin solution used in the coating step. Conjugated gelatin/Thai silk fibroin scaffolds obtained from gelatin solution with low concentration showed fiber-like structure while the scaffolds obtained from gelatin solution with high concentration showed more membrane-like structure. At 0.5wt%

gelatin concentration, gelatin conjugation could form fibers inside the porous structure of Thai silk fibroin scaffolds as present in Figure 4.3(c)-4.3(d). At 1.0wt% and 1.5wt% gelatin concentration used, conjugated gelatin/Thai silk fibroin scaffolds showed more thin-wall formation inside the porous structure of Thai silk fibroin scaffolds as presented in Figure 4.3(e)-(f) and (g)-(h), respectively. This was due to the more amount of gelatin incorporated in the scaffolds. In addition, the morphology of conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.5wt% gelatin concentration was found to be heterogeneous.

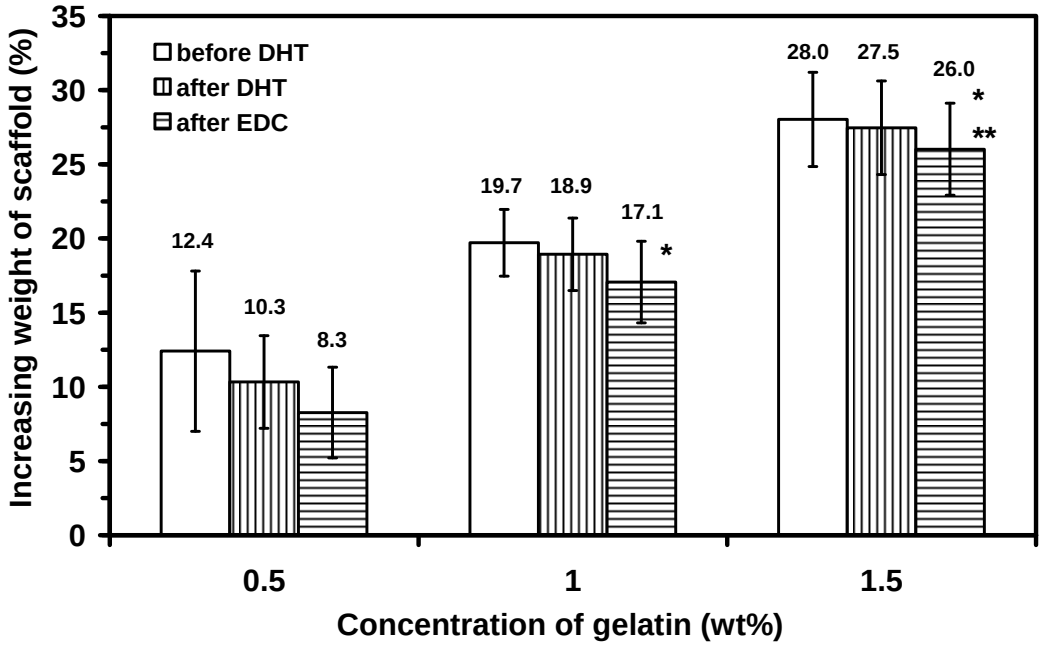

Figure 4.2 Increasing weight of Thai silk fibroin scaffolds in three different steps of gelatin conjugating when using various concentration of gelatin.

* and ** represent the significant difference ($p<0.05$) relative to the same step at the concentration of gelatin of 0.5wt% and 1.0wt%, respectively.

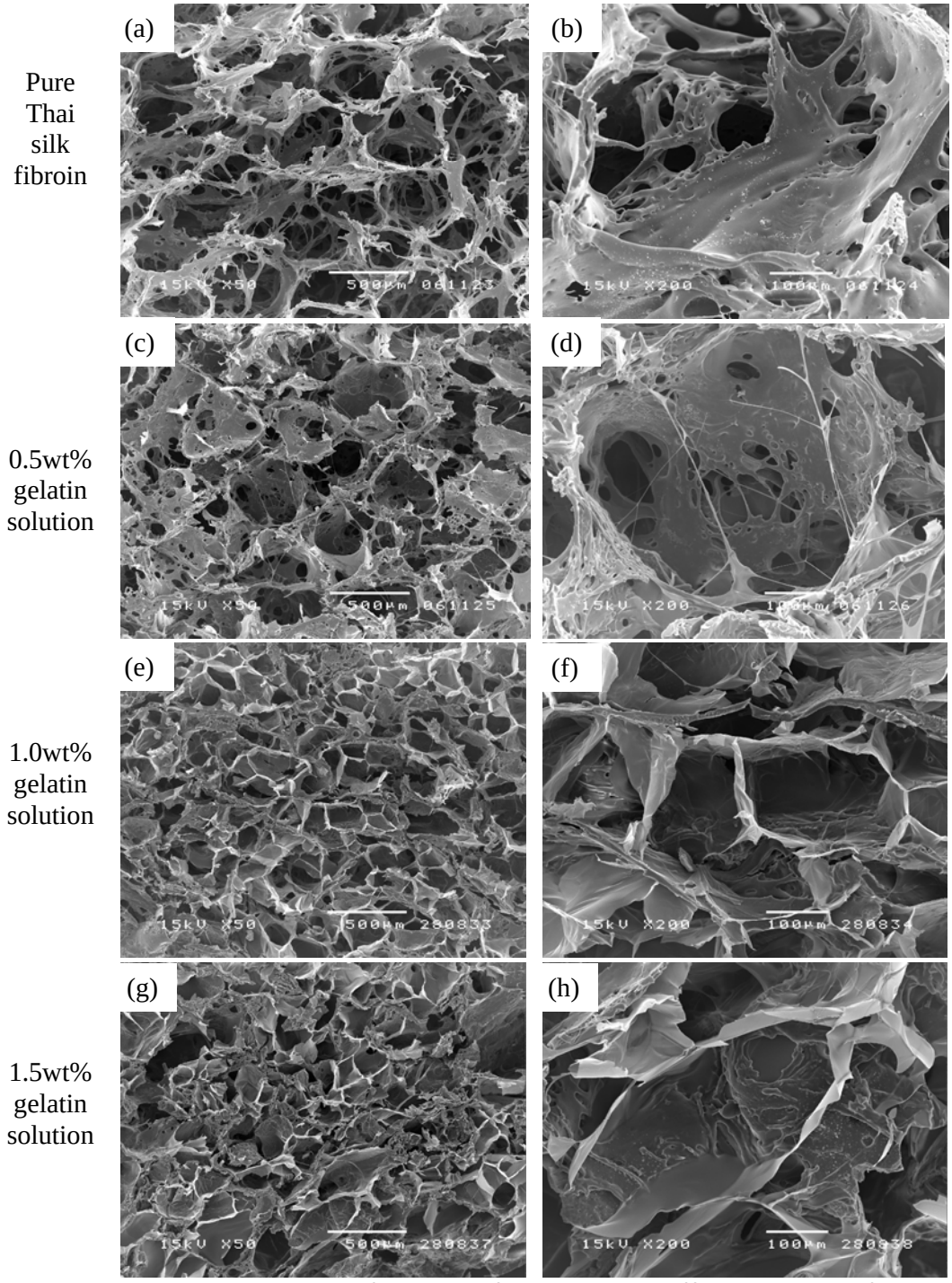

Figure 4.3 SEM micrographs of Thai silk fibroin based-scaffolds: Thai silk fibroin scaffolds (a-b), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration (c-d), 1.0wt% gelatin concentration (e-f) and 1.5wt% gelatin concentration (g-h).

4.1.2 *In vitro* cell proliferation tests

Figure 4.4 presented the numbers of MSCs on conjugated gelatin/Thai silk fibroin scaffolds after 6, 72 and 120 hour of the culture. At 6 hour after seeding, the number of cells on conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% and 1.5wt% gelatin concentration was higher than that of Thai silk fibroin and conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration. After 72 hour of seeding, the number of cells tended to increase in all scaffolds except in Thai silk fibroin scaffolds. The highest number of MSCs at 120 hours after seeding was noticed in conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration. It was observed that MSCs proliferated better on the conjugated gelatin/Thai silk fibroin scaffolds compared to pure silk fibroin scaffolds. This could be the result of arginine-glycine-aspartic (RGD) sequence contained in gelatin that could promote cell proliferation [44]. However, the number of cells tended to decrease in conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.5wt% gelatin concentration after 120 hour of seeding. This might be due to the morphology of scaffolds. More gelatin contents formed thin-wall which might obstruct the cell migration into the scaffolds. Hence, the suggested concentration of gelatin solution for conjugating onto Thai silk fibroin scaffolds should be less than 1.5wt%. Conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration and 1.0wt% gelatin concentration were then selected for biological characterization.

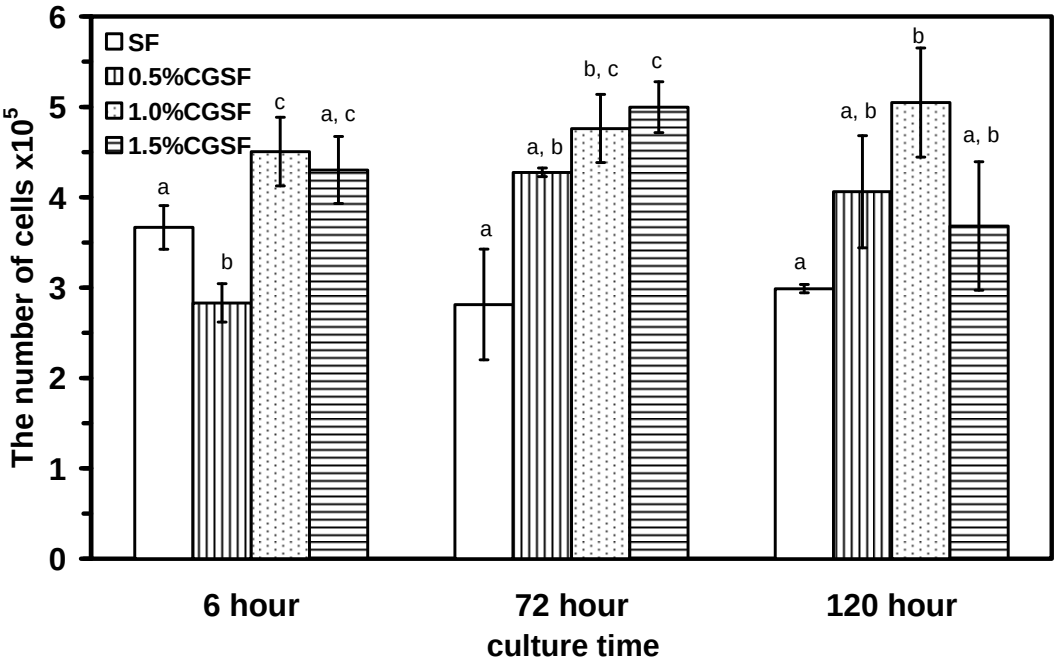

Figure 4.4 The number of bone-marrow derived mesenchymal stem cells on conjugated gelatin/Thai silk fibroin scaffolds after 6, 72 and 120 hour of culture (seeding: $5x10^5$ cells/scaffold).

a, b, c and d represent the significant difference within same culture period ($p<0.05$).

(The results with the same alphabet indicate that they are not significantly different)

4.2 Effects of hydroxyapatite deposition on Thai silk fibroin-based scaffolds

4.2.1 Soaking time in each solution

During alternate soaking process using calcium chloride and disodium hydrogen phosphate solutions, hydroxyapatite was expected to form and deposit into the Thai silk fibroin and conjugated gelatin/Thai silk fibroin scaffolds according to the reaction belows [20].

$$10CaCl_2 + 6Na_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12NaCl + 8HCl$$

The increasing weight of scaffold after alternate soaking process was determined and considered as the amount of hydroxyapatite deposited.

The increasing weight of hydroxyapatite in Thai silk fibroin scaffolds after 4 cycles of alternate soaking at three various soaking time in each solution was presented in Figure 4.5. The weight of hydroxyapatite deposited into scaffolds was increased upon the increasing time of soaking from 10 to 30 minutes. As increasing soaking time in each solution from 10 to 20 minutes, the weight of hydroxyapatite deposited in scaffolds increased from about 90% to 126%. When 30 minute of soaking time was applied, the highest amount of hydroxyapatite deposited in scaffolds was noticed. However, there was no significant difference in the amount of hydroxyapatite deposited into scaffolds when soaking time in each solution was changed from 20 to 30 minutes. Hence, the soaking time in each solution for 30 minutes was selected for further investigation in this study.

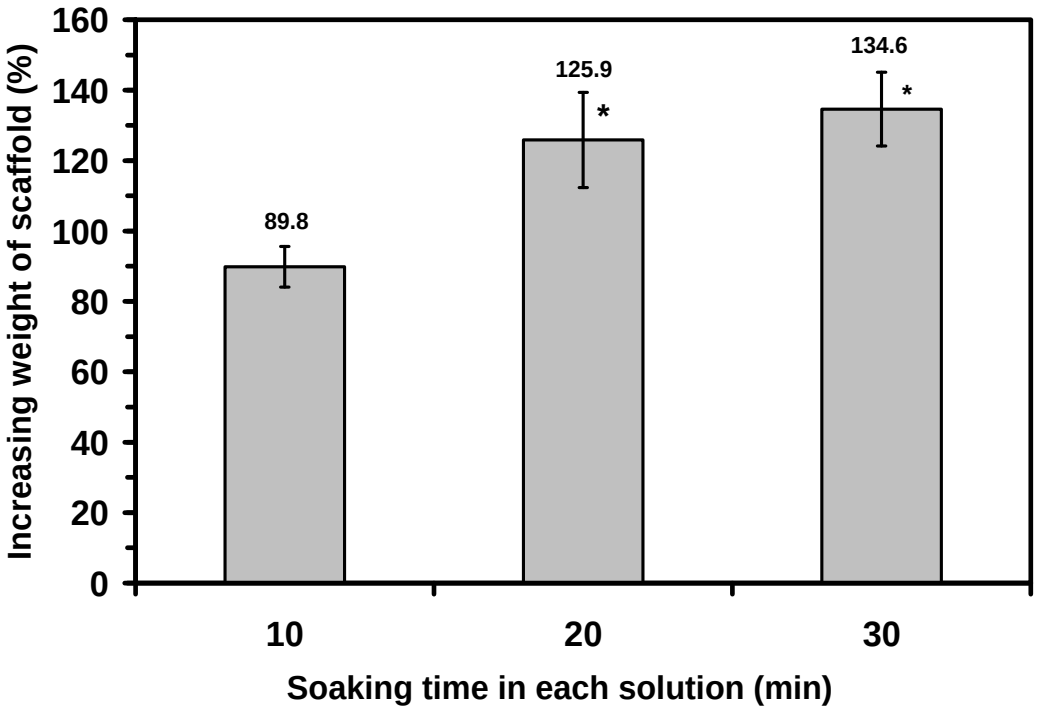

Figure 4.5 Increasing weight of hydroxyapatite in Thai silk fibroin scaffolds after 4 cycles of alternate soaking at three various soaking time in each solution (calcium chloride and disodium hydrogenphosphate solutions).

* represent the significant difference ($p<0.05$) relative to soaking time in each solution for 10 minute.

4.2.2 Cycles of alternate soaking

In this section, the effects of alternate soaking cycles on the morphology and properties of Thai silk fibroin-based scaffolds were reported. The soaking time in each solution was fixed at 30 minutes. The alternate soaking was performed for 1, 2, 4, 6 and 8 cycles. The ratio of protein (organic part) and hydroxyapatite (inorganic part) contained in obtained Thai silk fibroin-based scaffolds was investigated by the increasing weight of scaffolds after alternate soaking process. All types of Thai silk fibroin-based scaffolds prepared were listed in Table 4.2.

Table 4.2 List of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/ conjugated gelatin/Thai silk fibroin scaffolds prepared in this work.

Scaffold type	Number of alternate soaking	Notation
Hydroxyapatite/ Thai silk fibroin scaffolds	0	SF
	1	SF1
	2	SF2
	4	SF4
	6	SF6
	8	SF6
Hydroxyapatite/ conjugated gelatin/Thai silk fibroin scaffolds	0	CGSF
	1	CGSF1
	2	CGSF2
	4	CGSF4
	6	CGSF6
	8	CGSF8

4.2.2.1 Morphology of scaffolds

The ratio of organic to inorganic in hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds as a function of alternate soaking cycles was shown in Figure 4.6. After the first cycle of alternate soaking, the ratio of organic to inorganic in hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds was 64:36 and 71:29, respectively. The ratio of organic to inorganic of both types of scaffolds increased with an increasing in the number of alternate soaking cycles. After 4 cycles of alternate soaking, the ratio of organic to inorganic in Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds was 44:56 and 48:52, respectively. Hydroxyapatite deposited after 6 and 8 cycles of alternate soaking in conjugated gelatin/Thai silk fibroin scaffolds were similar to that deposited after 4 cycles of alternate soaking. Meanwhile, hydroxyapatite deposited after 6 and 8 cycles of alternate soaking in Thai silk fibroin scaffolds was increased about 1.2 and 1.3 folds of hydroxyapatite deposited after 4 cycles of alternate soaking. This result indicated that the highest amount of hydroxyapatite accumulation into scaffolds could be noticed after the first cycle of alternate soaking in calcium chloride and disodium hydrogenphosphate solutions.

Morphology of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/ conjugated gelatin/Thai silk fibroin scaffolds revealed by SEM photographs illustrated in Figure 4.7 and Figure 4.8, indicated the accumulated hydroxyapatite throughout the scaffolds. The hydroxyapatite crystal aggregation increased as increasing the number of alternate soaking cycles while the pore volume of scaffolds was decreased. The deposition of hydroxyapatite in Thai silk fibroin scaffolds was greater than in conjugated gelatin/Thai silk fibroin scaffolds. This could be a result from morphology. Gelatin conjugation caused less pore volume of scaffolds which could limit the deposition of hydroxyapatite into the scaffolds. The hydroxyapatite deposited on the outer surface of the scaffolds was more than that deposited inside the scaffolds. After 6 and 8 cycles of alternate soaking, the more hydroxyapatite deposition led to a thick layer of hydroxyapatite fully filled the porous structure.

Therefore, the appropriate scaffolds which show suitably porous morphology were hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds obtained form 4 cycles of alternate soaking in calcium chloride and disodium hydrogenphosphate solutions**.**

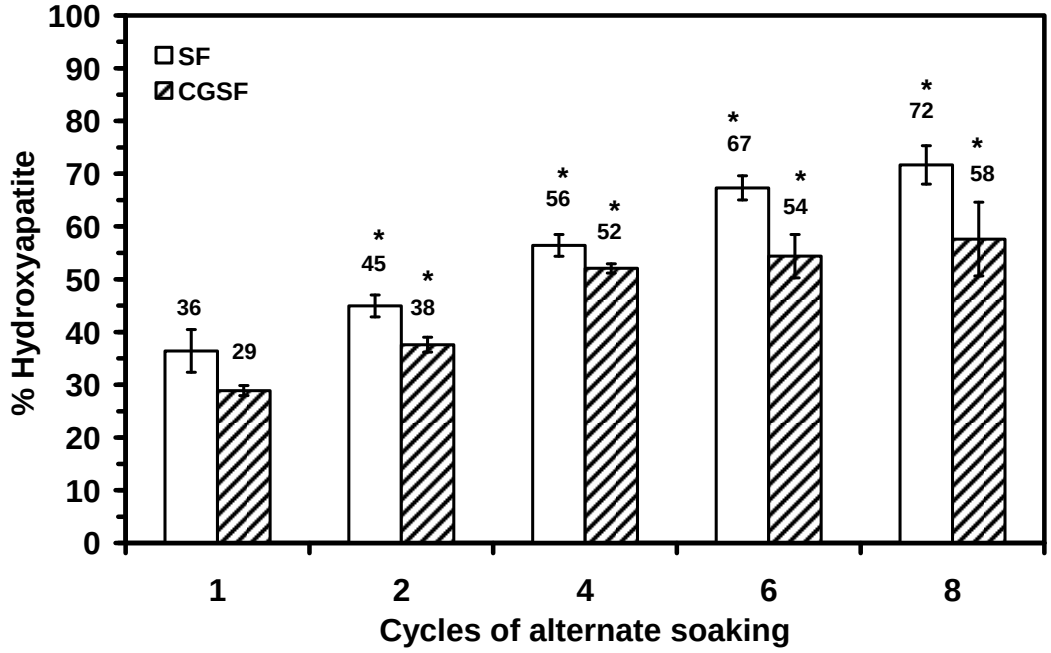

Figure 4.6 Percentage of hydroxyapatite contents in hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds as a function of alternate soaking cycles.

* represent the significant difference ($p<0.05$) relative to each scaffold after the first cycle of alternate soaking.

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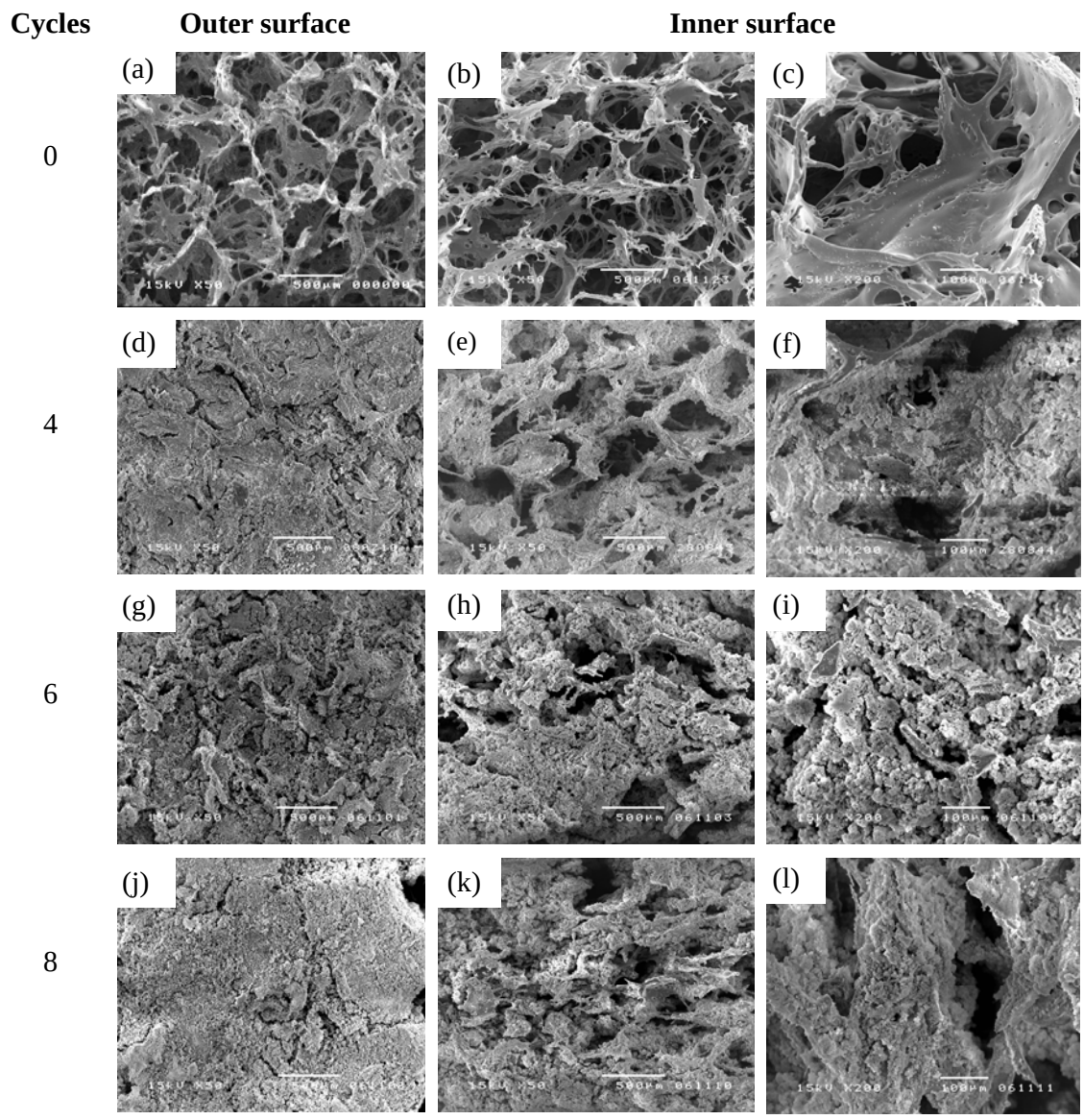


Figure 4.7 SEM micrographs of Thai silk fibroin scaffolds (a)-(c), hydroxyapatite/ Thai silk fibroin scaffolds prepared from (d)-(f) 4 cycles, (g)-(h) 6 cycles and (i)-(l) 8 cycles of alternate soaking.

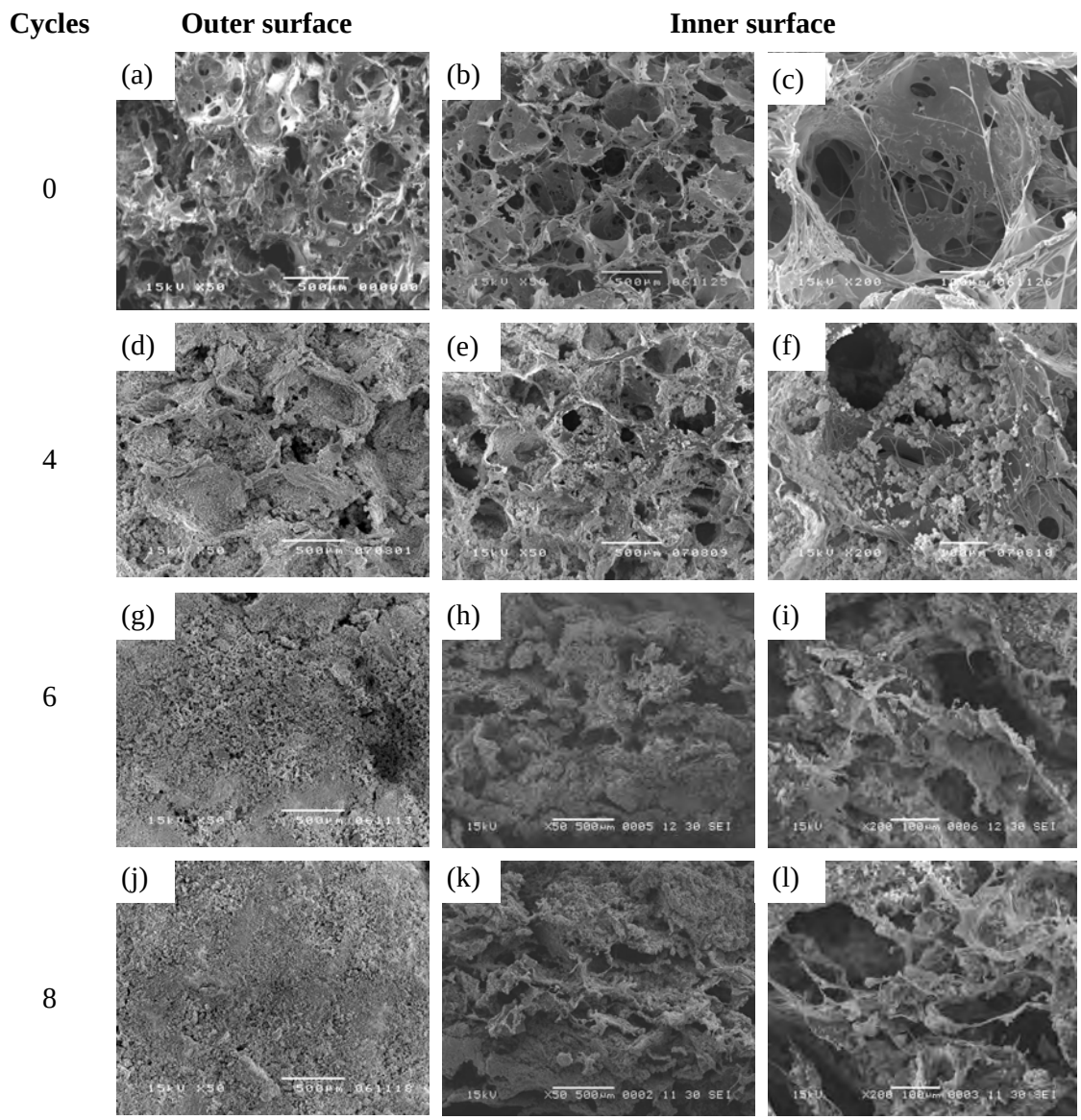


Figure 4.8 SEM micrographs of conjugated gelatin/Thai silk fibroin scaffolds (a)-(c), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from (d)-(f) 4 cycles, (g)-(h) 6 cycles and (i)-(l) 8 cycles of alternate soaking.

4.2.2.2 Compressive modulus of scaffolds

The compressive modulus of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds shown in Figure 4.9 indicated that the compressive modulus of pure Thai silk fibroin scaffold was 375±88 kPa. Our previous report on this value by Chamchongkaset *et.al*. [46] was 263±62 kPa. Kim U.J. et.al. [25] have also reported that the compressive modulus of salt-leached Japanese silk fibroin scaffolds was 770±50 kPa. It was higher than the compressive modulus in this reported. This could be because of the different source of *Bombyx mori* silkworm and testing condition. Our scaffolds were small (11 mm in diameter and 2 mm in height) comparing to those used in that report (12 mm in diameter and 10 mm in height). The compressive modulus of conjugated gelatin/Thai silk fibroin scaffolds was found to be higher than that of Thai silk fibroin scaffolds. This could be a result from the conjugation reaction by dehydrothermal and EDC treatments. Dehydrothermal brings about chemical bonding between the amino and carboxyl groups within molecules of polypeptide. Further treatment with EDC, the primary amines on the peptides formed a stable amide bond between the peptide of gelatin and silk fibroin [45]. After hydroxyapatite deposition, the compressive modulus of both types of scaffolds increased with increasing in the number of alternate soaking cycles. This was possibly due to the amount of hydroxyapatite accumulated in scaffolds which promoted the strength of the scaffolds. The results indicated that gelatin conjugation and hydroxyapatite deposition enhanced the compressive modulus of Thai silk fibroin scaffolds as reported by Chamchongkaset *et.al*. [46].

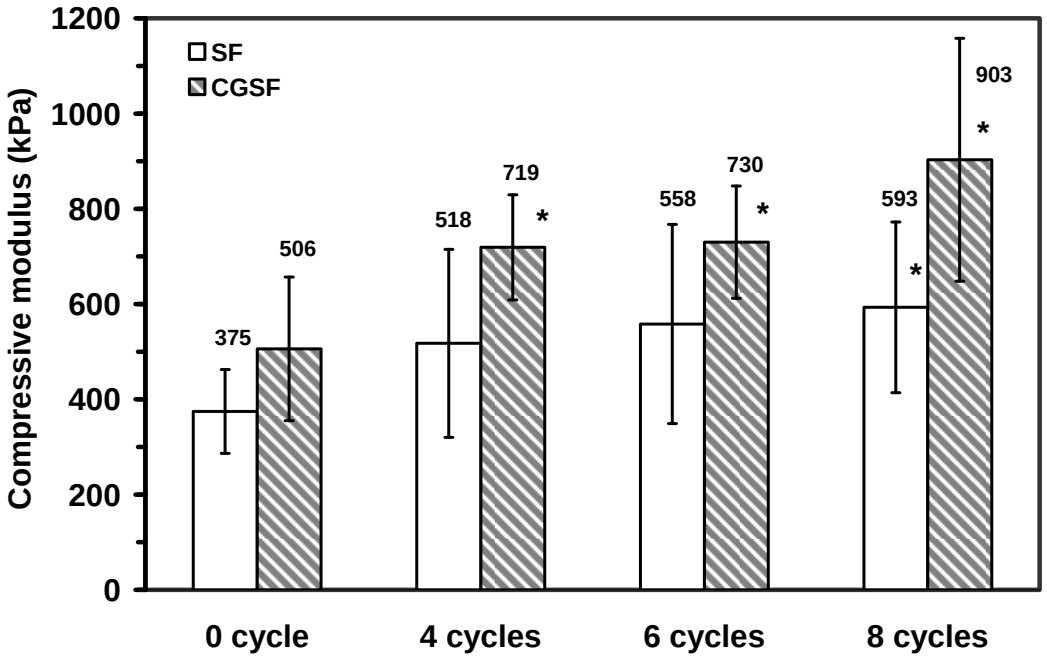


Figure 4.9 Compressive modulus of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0, 4, 6 and 8 cycles of alternate soaking.

* represent the significant difference ($p<0.05$) relative to each scaffold before alternate soaking (0 cycle).

4.2.2.3 Thermal properties

Figure 4.10 presented TGA thermograms of Thai silk fibroin scaffolds, hydroxyapatite/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking, conjugated gelatin/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking. The weight loss at temperature below 100°C was attributed to the moisture content in the scaffolds. The degradation temperature of hydroxyapatie/Thai silk fibroin scaffold and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold determined at 5% weight loss was about 230°C while that of Thai silk fibroin scaffold and conjugated gelatin/Thai silk fibroin scaffold was about 255°C. The thermograms illustrated that Thai silk fibroin scaffold and conjugated gelatin/Thai silk fibroin scaffold could completely degrade under oxygen atmosphere without any char left. This was due to the fact that the scaffolds contained only proteins. Meanwhile, the char yield of the scaffolds containing inorganic part, hydroxyapatie/Thai silk fibroin scaffold and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold, were noticed at approximately 52%.

This result indicated that the hydroxyapatie/Thai silk fibroin scaffold and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold contain 48% of protein and 52% of inorganic. This corresponded to the ratio of organic to inorganic in scaffolds previously discussed from the increasing weight of scaffolds.

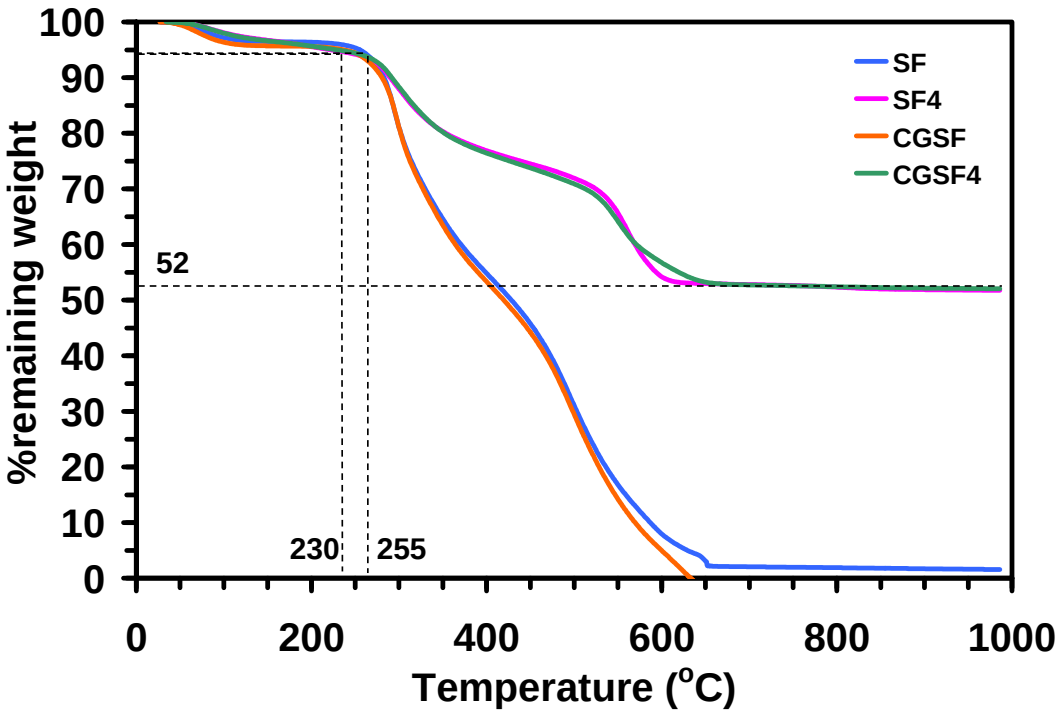


Figure 4.10 TGA thermograms of Thai silk fibroin scaffolds, hydroxyapatite/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking, conjugated gelatin/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking obtained under oxygen atmosphere.

4.2.3 Alternate soaking steps

4.2.3.1 Morphology of scaffolds

In this section, the effects of alternate soaking steps on the properties of Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds were investigated. Four cycles of the alternate soaking in calcium chloride solution and disodium hydrogenphosphate solution was performed with the soaking time of 30 minutes in each solution. Two different alternate soaking steps, i.e. different order of soaking solution, were compared as follows.

process 1 : First immersing in calcium chloride solution following by disodium hydrogenphosphate solution (Ca-P).

process 2 : First immersing in disodium hydrogenphosphate solution following by calcium chloride solution (P-Ca).

The increasing weight of hydroxyapatite in Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds using two different alternate soaking steps was presented in Figure 4.11. The increasing weight of hydroxyapatite deposited in conjugated gelatin/Thai silk fibroin scaffolds soaking first in disodium hydrogenphosphate solution was significantly higher than those soaking first in calcium chloride solution. This might be due to the charge of scaffolds. Conjugated gelatin/Thai silk fibroin scaffolds consisted of type A gelatin fiber conjugated onto the scaffold surface, resulting in a positive-charged surface. In this study, type A gelatin solution at pH7 presented cationic charges [7]. The surface of conjugated gelatin/Thai silk fibroin scaffolds which were partly positive could interact with phosphate solution (anionic charge) and repulse calcium solution (cationic charge). Hence, soaking conjugated gelatin/Thai silk fibroin scaffolds in disodium hydrogenphosphate solution followed by calcium chloride solution could induce more hydroxyapatite deposition than those soaking in calcium chloride solution followed by disodium hydrogenphosphate solution.

For the case of Thai silk fibroin scaffolds soaking first in disodium hydrogenphosphate solution tended to result in a slightly more amount of hydroxyapatite deposited comparing to those soaking first in calcium chloride solution. However, there was no significant difference in the increasing weight of

hydroxyapatite between soaking first in disodium hydrogenphosphate solution and soaking first in calcium chloride solution. The result indicated that the step of alternate soaking influenced the amount of deposited hydroxyapatite.

The morphology of hydroxyapatite crystals deposited in Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds was shown in Figure 4.12. It was found that the internal surface of all types of scaffolds were fully covered with hydroxyapatite crystals. The mineralized crystals were flower-like. The size of hydroxyapatite crystals was about 5 μm. Difference in the step of alternate soaking did not affect the morphology of deposited hydroxyapatite crystals.

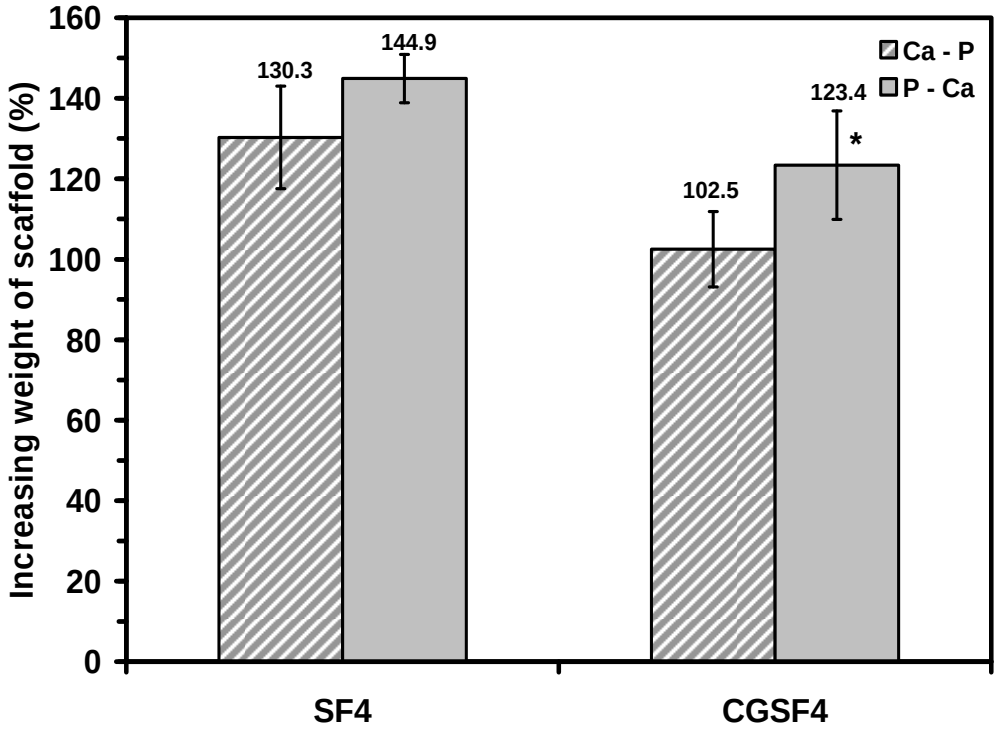


Figure 4.11 Increasing weight of hydroxyapatite deposited in Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds using two different alternate soaking steps; first immersing in calcium chloride solution following by disodium hydrogenphosphate solution (Ca-P) and first immersing in disodium hydrogenphosphate solution following by calcium chloride solution (P-Ca).
* represent the significant difference ($p<0.05$) relative to the same scaffold soaking first in calcium chloride solution (Ca-P).

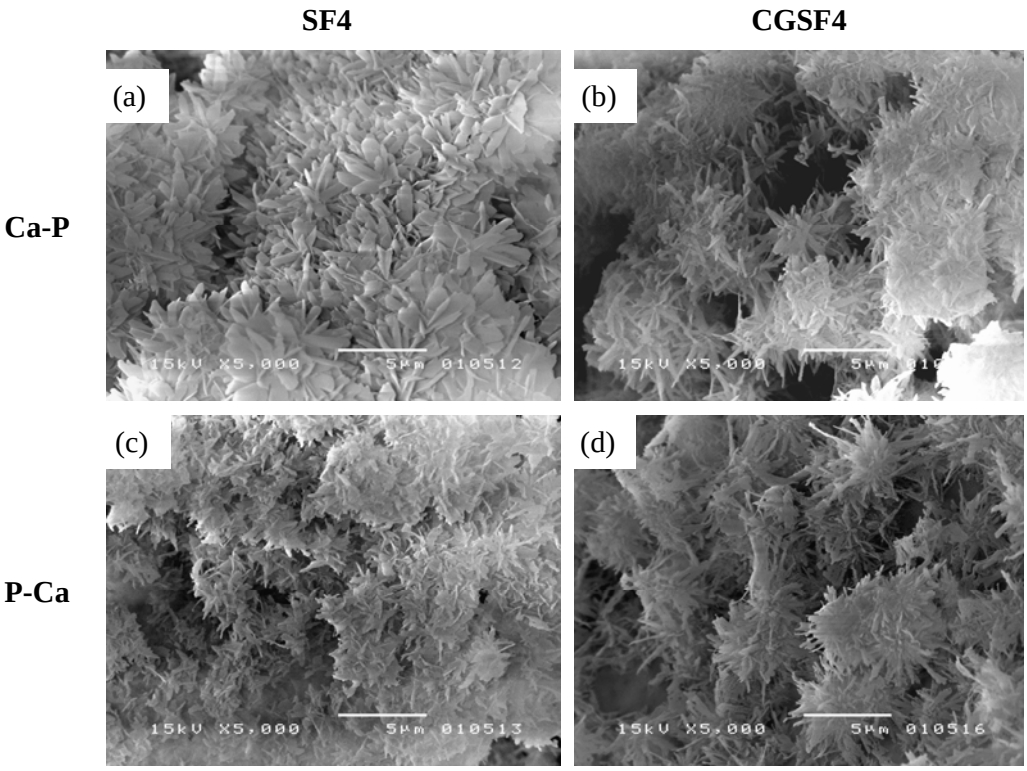


Figure 4.12 SEM micrographs of hydroxyapatite crystals in Thai silk fibroin scaffolds soaking in calcium chloride solution followed by disodium hydrogenphosphate solution process (a), conjugated gelatin/Thai silk fibroin scaffolds soaking in calcium chloride solution followed by disodium hydrogenphosphate solution process (b), Thai silk fibroin scaffolds soaking in disodium hydrogenphosphate solution followed by calcium chloride solution process (c), and conjugated gelatin/Thai silk fibroin scaffolds soaking in disodium hydrogenphosphate solution followed by calcium chloride solution process (d).

4.2.3.2 Compressive modulus of scaffolds

The compressive modulus of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from two different alternate soaking steps was shown in Figure 4.13. It was noticed that the compressive modulus of hydroxyapatite/Thai silk fibroin scaffolds prepared from soaking first in calcium chloride solution followed by disodium hydrogenphosphate solution process was higher than that soaking in disodium hydrogenphosphate solution followed by calcium chloride solution process about 1.8 folds. However, there was no significant difference in the compression modulus of Thai silk fibroin scaffolds between soaking first in calcium chloride solution and soaking first in disodium hydrogenphosphate solution. For the case of hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds, the compressive modulus of hydroxyapatite/ conjugated gelatin/Thai silk fibroin scaffolds soaking first in calcium chloride solution were similar to those soaking first in disodium hydrogenphosphate solution.

As comparing the compressive modulus between hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds, the compressive modulus of hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds soaking first in calcium chloride solution and disodium hydrogenphosphate solution was higher than that of hydroxyapatite/Thai silk fibroin scaffolds. This result corresponded to a recent study by Chamchongkaset *et.al.* [46]. They first reported that gelatin conjugation onto the silk fibroin scaffolds enhanced the compressive modulus of silk fibroin scaffolds.

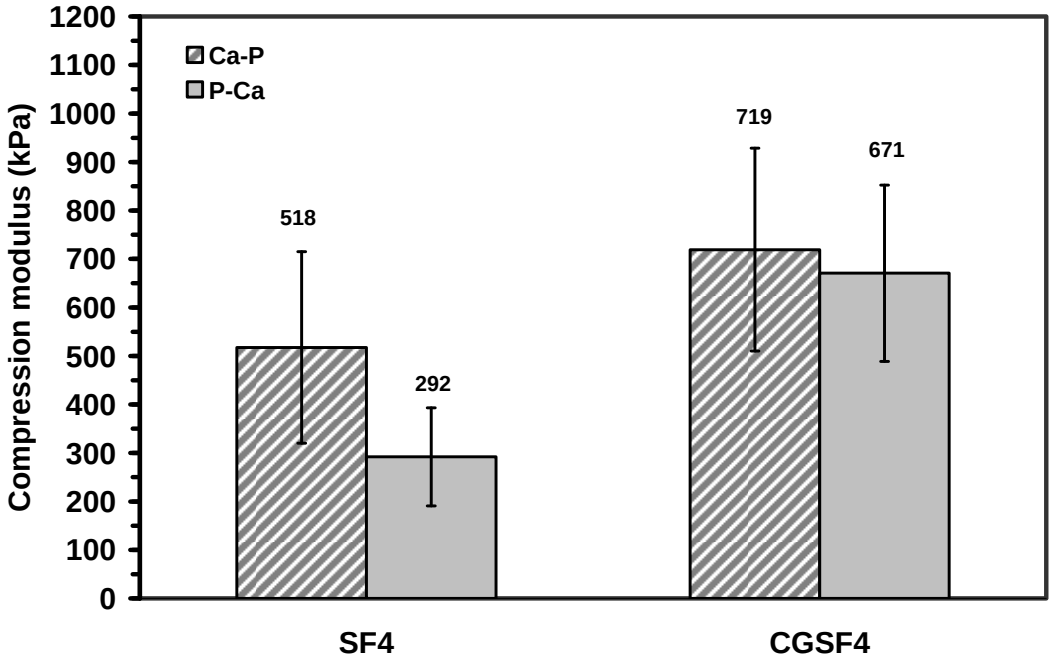


Figure 4.13 Compressive modulus of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from two different alternate soaking steps; first immersing in calcium chloride solution following by disodium hydrogenphosphate solution (Ca-P) and first immersing in disodium hydrogenphosphate solution following by calcium chloride solution (P-Ca). * represent the significant difference ($p<0.05$) relative to the same scaffold soaking first in calcium chloride solution (Ca-P).

4.2.3.3 Elemental analysis

The results on element analysis of hydroxyapatite/Thai silk fibroin scaffolds (SF4) and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds (CGSF4) prepared from two different alternate soaking steps examined by EDX were shown in Table 4.3. This result showed that there were both organic and inorganic elements found on the surface of scaffolds including carbon, oxygen, calcium, phosphorous, sodium and chloride. The organic parts were definitely from Thai silk fibroin and gelatin components. The Ca/P ratio of hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds soaking first in calcium chloride solution were 1.71 and 1.51, respectively. These Ca/P ratios were in the range apatite phase with a Ca/P ratio close to that of bone mineral. Wang *et.al.* [47] has reported that the Ca/P ratio of representative bone minerals in humans and animals varies according to nutritional status and age, ranging from 1.15 to 1.70. Meanwhile, the Ca/P ratio of scaffold prepared from soaking first in disodium hydrogenphosphate solution was about 2.4 which did not correspond to the Ca/P ratio of apatite phase.

Comparing the amount of sodium and chloride residue from the two different alternative soaking steps, the amount of chloride residue of both scaffolds soaking first in calcium chloride solution were lower than those soaking first in disodium hydrogenphosphate solution. Meanwhile, the amount of sodium residue of both scaffolds soaking first in calcium chloride solution was more than that soaking first in disodium hydrogenphosphate solution. This could be because of the last immersing solution used in alternate soaking process. In the case of scaffolds soaking first in calcium chloride solution, the last immersing solution used was disodium hydrogenphosphate solution which could lead to more amount of sodium residue on scaffold surface. For the other step, the last immersing solution used was calcium chloride solution. This could lead to more chloride residue left on scaffold surface.

Therefore, the suitable alternate soaking step selected for scaffold preparation for further investigation in this study was the process of soaking Thai silk fibroin-based scaffolds in calcium chloride solution first, followed by disodium hydrogenphosphate solution.

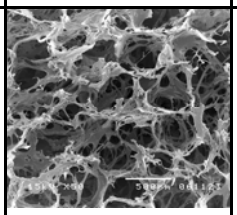
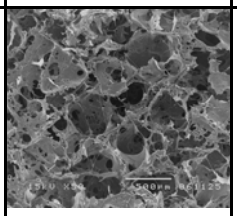
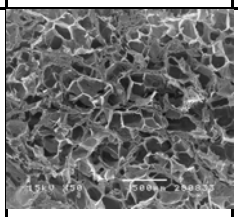
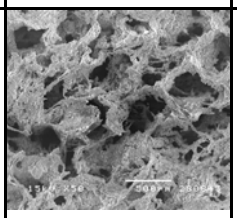
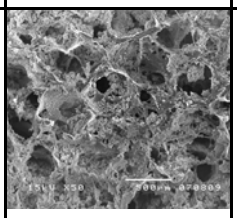
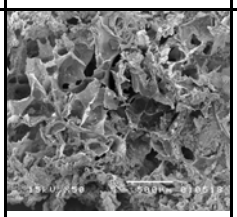
Table 4.3 Element component in hydroxyapatite/Thai silk fibroin scaffolds and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from two different alternate soaking steps; first immersing in calcium chloride solution following by disodium hydrogenphosphate solution (Ca-P) and first immersing in disodium hydrogenphosphate solution following by calcium chloride solution (P-Ca).

Scaffold	**SF4**		**CGSF4**	
Alternate soaking step	**Ca-P**	**P-Ca**	**Ca-P**	**P-Ca**
C	6.00	13.12	7.60	8.68
O	38.30	31.67	37.87	28.95
Na	4.43	1.92	6.16	0.36
P	18.64	11.98	17.98	15.16
Cl	0.70	12.08	3.19	9.35
K	-	0.52	-	-
Ca	31.93	28.71	27.21	37.50
Total	100.00	100.00	100.00	100.00
Ca:P	1.71	2.40	1.51	2.47

4.3. Bioloical properties of the scaffolds

The appropriate Thai silk fibroin-based scaffolds were selected to test their biological properties. Table 4.4 summarized the properties of all scaffolds which were selected for *in vitro* cell culture. Two main criteria used for selecting these scaffolds are porous morphology with interconnected network and appropriate compressive modulus. Porous morphology with interconnected network is needed for cell penetration while high compressive modulus is an essential property to ensure that scaffolds can absorb forces when they are implanted into the body.

Table 4.4 Properties of Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration, hydroxyapatite/ Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration for *in vitro* cell culture

Scaffold Type	**Organic: Inorganic**	**Morphology**	**Properties of scaffold**
Thai silk fibroin scaffold; SF	100 : 0		Pore size: 561±44 μm Density: 0.081±0.005 mg/mm^3 Porosity: 91.9±0.5 % Compressive modulus: 375±88 kPa
Conjugated gelatin/ Thai silk fibroin scaffold prepared from 0.5wt% gelatin concentration; 0.5% CGSF	100 : 0		Pore size: 485±42μm Density: 0.093±0.006 mg/mm^3 Porosity: 90.7±0.6 % Compressive modulus: 506±127 kPa
Conjugated gelatin/Thai silk fibroin scaffold prepared from 1.0wt% gelatin concentration; 1.0%CGSF	100 : 0		Pore size: 243± 29 μm Density: 0.105±0.013 mg/mm^3 Porosity: 89.5±1.3 % Compressive modulus: 544±131 kPa
Hydroxyapatite/Thai silk fibroin scaffold; SF4	44±2 : 56±2		Pore size: 300±58 μm Density: 0.130±0.009 mg/mm^3 Porosity: 87.0±0.9 % Compressive modulus: 518±197 kPa
Hydroxyapatite/conjugated gelatin/ Thai silk fibroin scaffold prepared from 0.5wt% gelatin concentration; 0.5%CGSF4	48±1 : 52±1		Pore size: 286±82 μm Density: 0.146±0.009 mg/mm^3 Porosity: 85.4±0.9 % Compressive modulus: 710±111 kPa
Hydroxyapatite/conjugated gelatin/ Thai silk fibroin scaffold prepared from 1.0wt% gelatin concentration; 1.0%CGSF4	49±1 : 51±1		Pore size: 163±40 μm Density: 0.182±0.010 mg/mm^3 Porosity: 81.8±1.0 % Compressive modulus: 650±90 kPa

4.3.1 *In vitro* cell proliferation tests

MSCs were cultured on Thai silk fibroin-based scaffolds, listed in Table 4.4, in proliferating medium (α-MEM, 15% FBS, 100 U/ml penicillin/streptomycin) for 6 hour, 3 and 5 days after seeding. The number of MSCs attached and proliferated on scaffolds, analyzed by DNA assay, was presented in Figure 4.14. The result showed that the cells could attach better on conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration than on the others. The number of cells were gradually increased in all organic (protein) scaffolds along 5 days of the culture. Among organic/inorganic scaffolds, the number of cells in scaffolds with hydroxyapatite deposited did not tend to obviously increase. This might be due to the morphology of the scaffolds. More hydroxyapatite and gelatin wall might obstruct the cell migration into the scaffolds. Another reason could be a limit of the mass transfer in the scaffolds. In static culture, oxygen and nutrients might not be well supplied into the scaffolds, resulting in non-proliferation. The number of cells in Thai silk fibroin scaffolds conjugated with gelatin were higher than that in pure Thai silk fibroin scaffolds. This was due to the RGD sequence in gelatin molecules that was reported to promote cell adhesion and proliferation [48]. Comparing two conjugated gelatin/Thai silk fibroin scaffolds, it was observed that the more amount of gelatin conjugated in scaffolds (1.0% CGSF) could result in the higher number of proliferated MSCs.

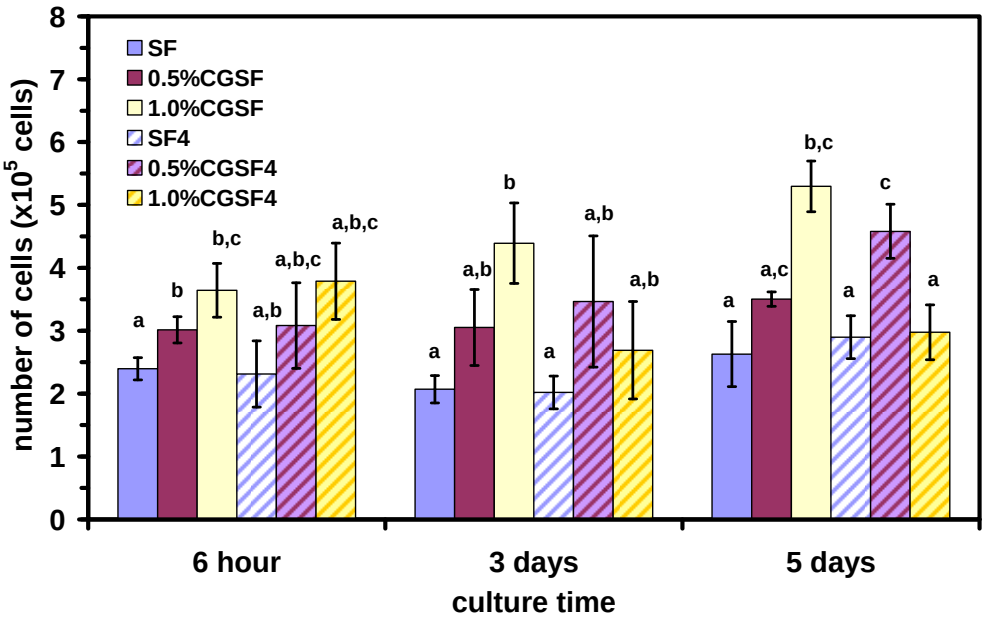


Figure 4.14 The number of MSCs on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentrationafter 6 hour, 3 and 5 days of seeding (seeding: $5x10^5$ cells/scaffold).
a, b and c represent the significant difference within same culture period ($p<0.05$).
(The results with the same alphabet indicate that they are not significantly different)

4.3.2 *In vitro* cell differentiation tests

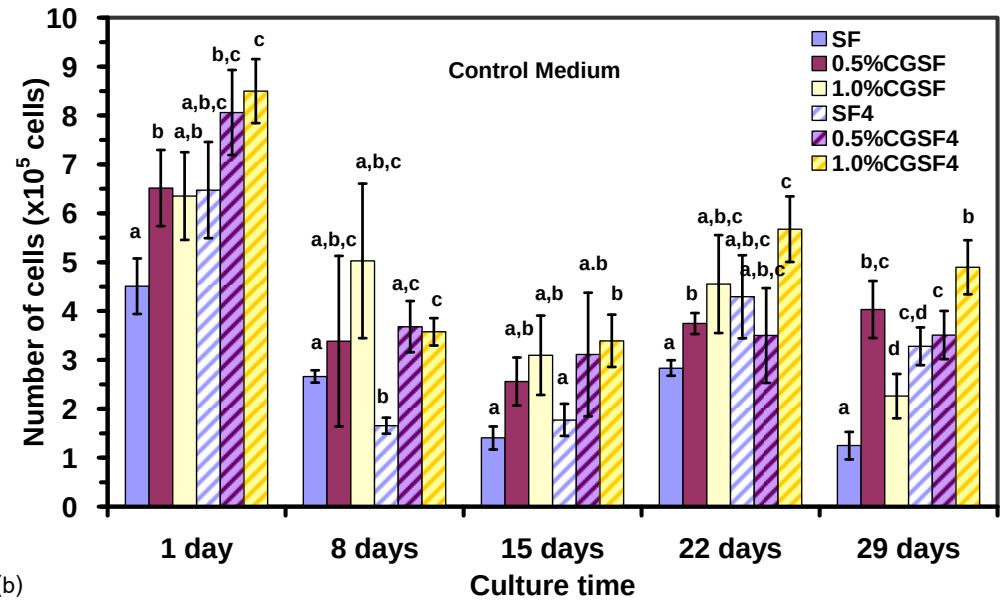
MSCs were cultured on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration in control medium (CM : α-MEM, 10% FBS) and osteogenic medium (OM : α-MEM, 10% FBS, 10 mM β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10 nM dexamethasone) for 1, 8, 15, 22 and 29 days after seeding. The number of cells on scaffolds along the osteogenic culture, analyzed by DNA assay, was presented in Figure 4.15(a) and (b). After 1 day of seeding, the number of MSCs attached on hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration was more than that on the other scaffolds. Thai silk fibroin scaffolds showed the least attached cells. This might be due to the hydrophobic property of silk fibroin. It caused the least swelling so that the cells could not attach well on Thai silk fibroin scaffolds. The number of cells after 8 days of seeding in both culture mediums was lower than the number of cells initially attached. This indicated the death of some cells on scaffolds. It was possibly due to the mass transfer limit in static culture of scaffolds as previously described. As MSCs were seeded into the scaffolds by agitation seeding, this led to a lot of MSCs attached. When the medium was changed every two days during static culture, the dead cells and detached cells could be eliminated from scaffolds. In osteogenic medium, the number of MSCs on scaffolds were maintained during 29 days of culture.

ALP activities of MSCs cultured on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration for 1, 8, 15, 22 and 29 days in control medium and osteogenic medium were shown in Figure 4.16(a) and (b). From ALP results, it was found that ALP activity of MSCs cultured on all scaffolds in control medium was not obviously different along the culture period. In the case of MSCs cultured in osteogenic medium, the least ALP activity on all scaffolds was noticed after the first week of MSCs seeding. After the first week ALP activity of MSCs on all scaffolds was increased until the maximum value was observed after 22 days of culture, except

hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration (0.5%CGSF) that showed the peak of ALP after 29 days of culture. The result showed that very high ALP activity could be noticed in the scaffolds containing all three components (silk fibroin, gelatin, and hydroxyapatite). This might be the result of hydroxyaptite which was known to promote cell differentiation [49].

In term of calcium deposition of MSCs, calcium contents of MSCs cultured on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration for 1, 8, 15, 22 and 29 days in control medium and osteogenic medium were shown in Figure 4.17(a) and (b). In the case of MSCs cultured in control medium, the calcium contents were relatively low, compared to those in osteogenic medium. The calcium contents tended to be maintained in all scaffolds along the culture period. Considering MSCs cultured in osteogenic medium, the calcium contents tended to increase in most scaffolds when the culture time increased except Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration. After 29 days of seeding, the highest calcium contents was observed on conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration. It was interesting to note that pure protein scaffold without hydroxyapatite, i.e. conjugation gelatin/Thai silk fibroin scaffold prepared from 1.0wt% gelatin concentration (1.0%CGSF), could induce calcium deposition of MSCs as good as other scaffolds containing hydroxyapatite.

(a)



(b)

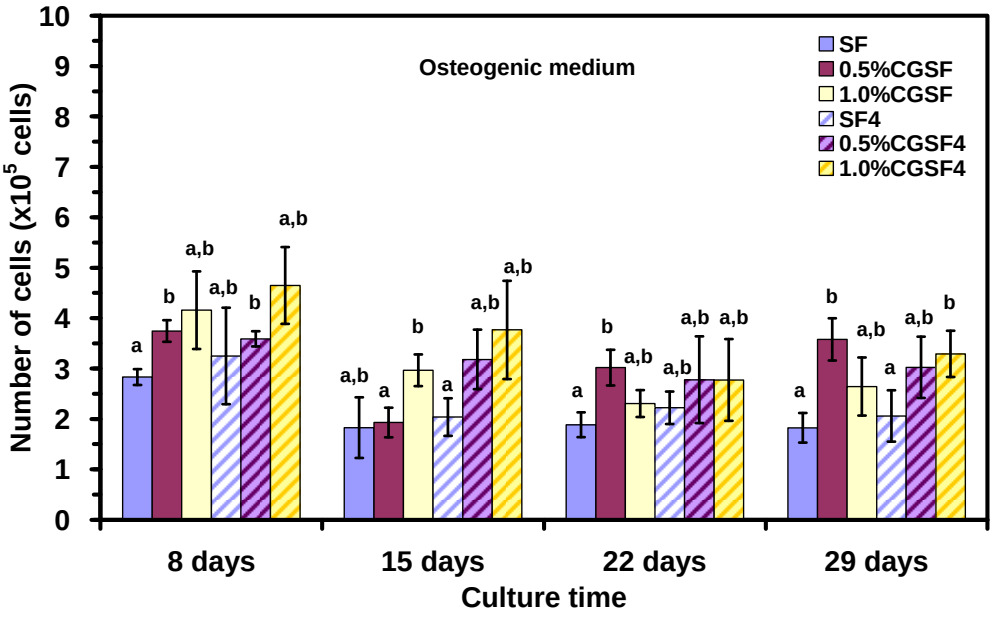
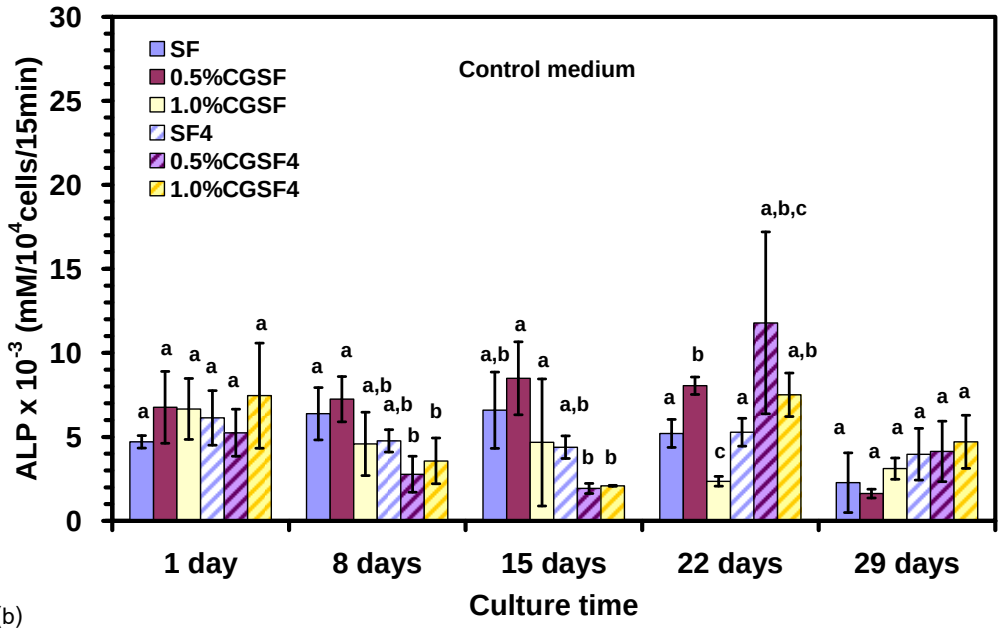


Figure 4.15 The number of MSCs cultured on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration in (a) control medium and (b) osteogenic medium after 1, 8, 15, 22 and 29 days after seeding (seeding: $1x10^6$ cells/scaffold).

a, b, c and d represent the significant difference within same culture period ($p<0.05$). (The results with the same alphabet indicate that they are not significantly different)

(a)



(b)

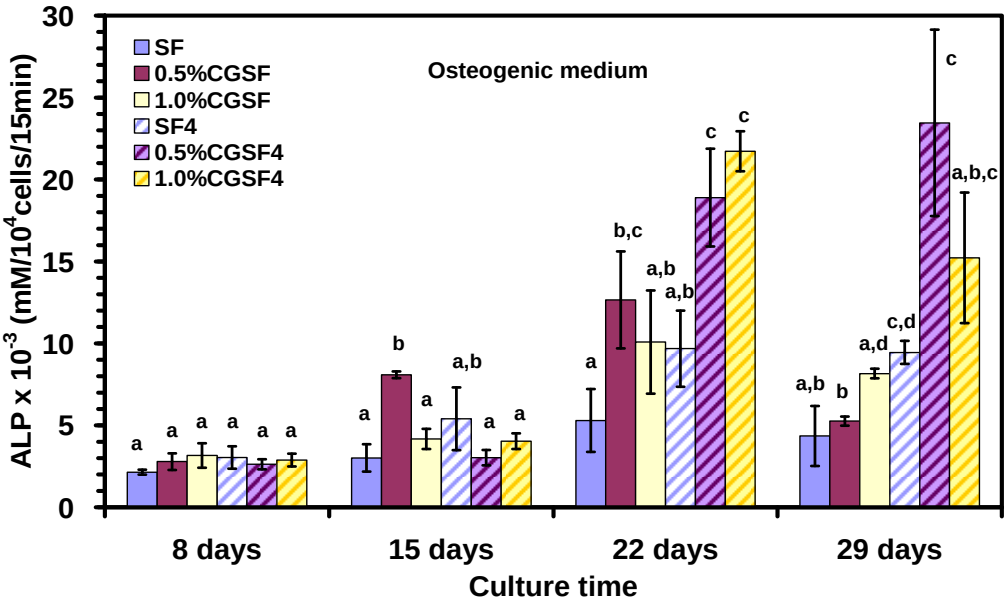
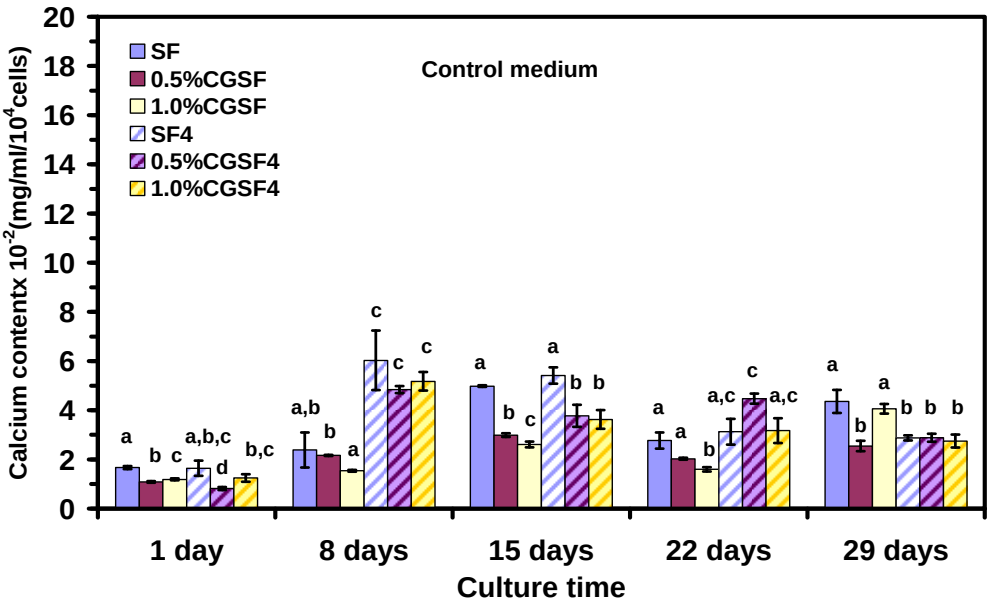


Figure 4.16 ALP activity of MSCs cultured on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration in (a) control medium and (b) osteogenic medium after 1, 8, 15, 22 and 29 days after seeding (seeding: $1x10^6$ cells/scaffold).

a, b, c and d represent the significant difference within same culture period ($p<0.05$). (The results with the same alphabet indicate that they are not significantly different)

(a)



(b)

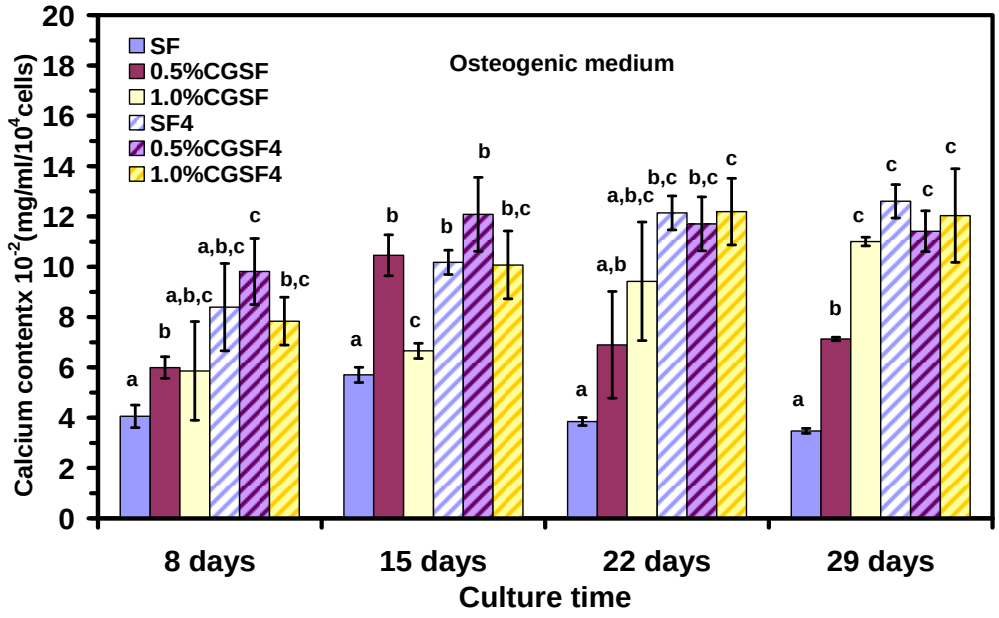


Figure 4.17 Calcium content of MSCs cultured on Thai silk fibroin scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration in (a) control medium and (b) osteogenic medium after 1, 8, 15, 22 and 29 days after seeding (seeding: $1x10^6$ cells/scaffold)

a, b and c represent the significant difference within same culture period ($p<0.05$).

(The results with the same alphabet indicate that they are not significantly different)

Figure 4.18 and 4.19 showed morphology of MSCs cultured on Thai silk fibroin scaffold, conjugated gelatin/Thai silk fibroin scaffold prepared from 0.5wt% and 1.0wt% gelatin concentration, hydroxyapatite/Thai silk fibroin scaffold, hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold prepared from 0.5wt% and 1.0wt% gelatin concentration after 29 days of seeding under control medium and osteogenic medium, respectively. Some round-shaped cells were observed on the scaffolds, especially on the ones containing hydroxyapatite. As reported by Nakamura [50] that osteoblasts are generally round in shape, this morphology could imply the osteogenic differentiation of MSCs. For the case of MSCs cultured in osteogenic medium (Figure 4.19), calcium crystals were seen on the surface of conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% and 1.0wt% gelatin concentration (0.5%CGSF and 1.0%CGSF). Especially in the conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration, a lot of calcium crystals were deposited homogenously throughout the scaffold surface. For the case of the scaffolds containing hydroxyapatite, calcium crystal formation was not clearly seen on the surface of scaffolds since the original surface of scaffolds was covered by deposited hydroxyapatite.

After 29 days of culture in osteogenic medium, all scaffolds were analyzed by energy dispersive x-ray spectrometer (EDX) to confirm the production of calcium from MSCs. EDX results of scaffold surfaces before cell culture were presented in Figure 4.20 while EDX results on the surface of the scaffold were shown in Figure 4.21, respectively. Table 4.5 summarized the amount of calcium and phosphate on Thai silk fibroin-based scaffolds before and after 29 days of MSCs culture. Calcium and phosphate contents were found in all protein scaffolds. Scaffold containing higher amount of gelatin (1%CGSF) showed higher calcium contents deposited compared to the one with lower amount of gelatin (0.5%CGSF). In the group of scaffolds containing hydroxyapatite, high amount of calcium deposited was observed in the scaffold with conjugated gelatin. The EDX results corresponded to the calcium content reported in Figure 4.17.

The results on osteogenic differentiation test indicated that, among six types of scaffolds, conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration (1.0%CGSF), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% gelatin concentration (0.5%CGSF4) and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration (1.0%CGSF4) have high osteoconductive potential. Particular amount of conjugated gelatin in Thai silk fibroin scaffolds could promote osteogenic differentiation of MSCs, comparable to Thai silk fibroin-based scaffolds containing hydroxyapatite.

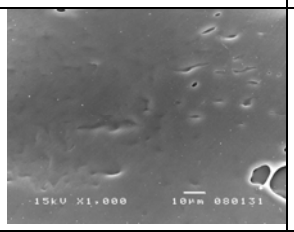
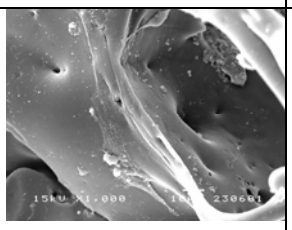
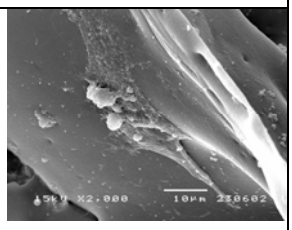
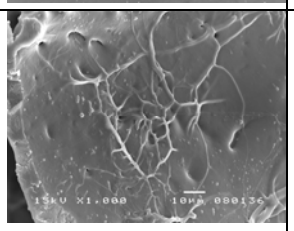
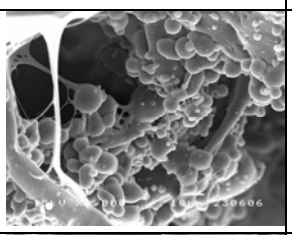
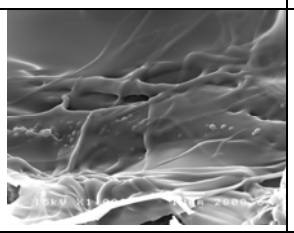
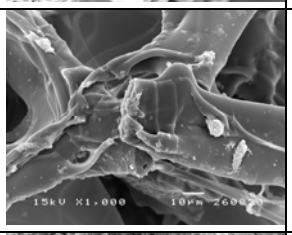
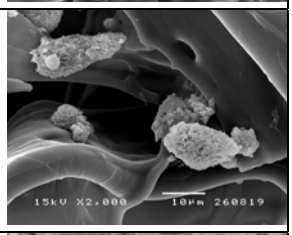
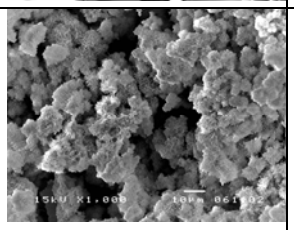
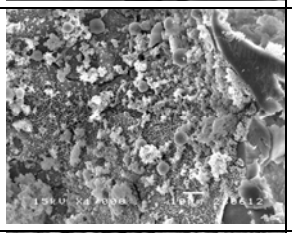
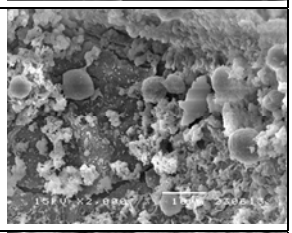
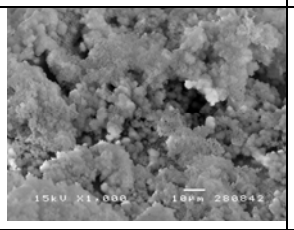
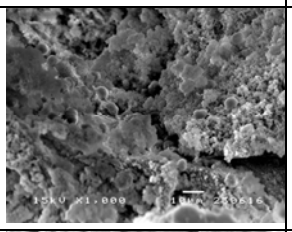
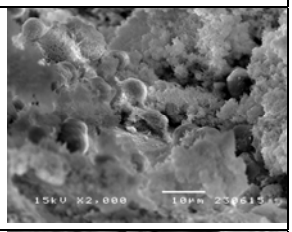
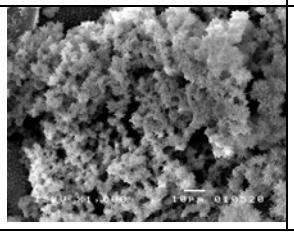
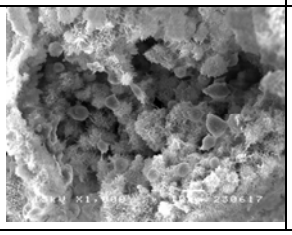
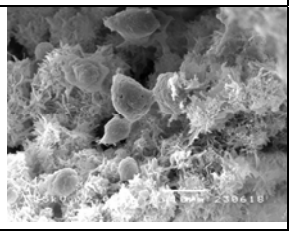
Scaffold type	Before culture	After culture in control medium	
	Scale bar : –10μm	Scale bar : –10μm	Scale bar : ——10μm
SF			
0.5%CGSF			
1.0%CGSF			
SF4			
0.5%CGSF4			
1.0%CGSF4			

Figure 4.18 Morphology of MSCs cultured on Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% (1.0%CGSF4) gelatin concentration for 29 days in control medium.

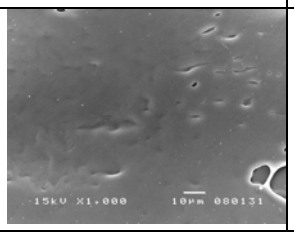
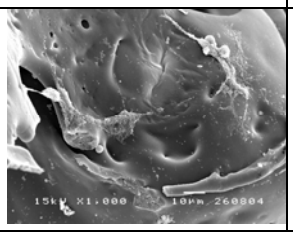
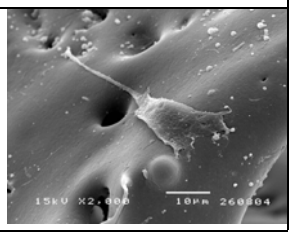
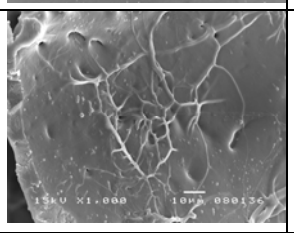
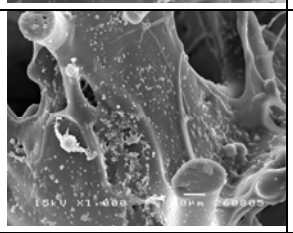
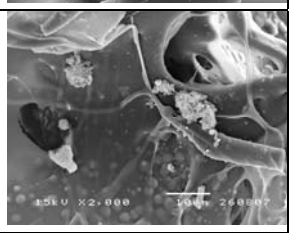
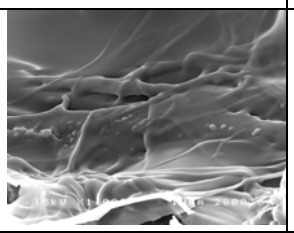
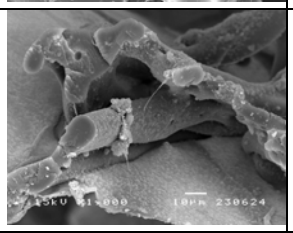
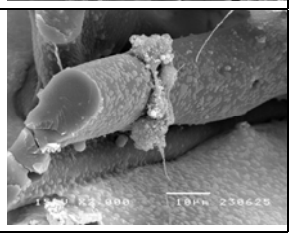
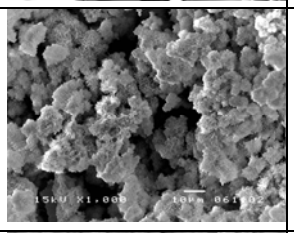
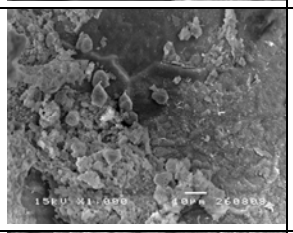
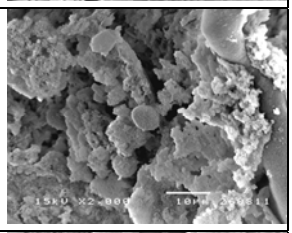
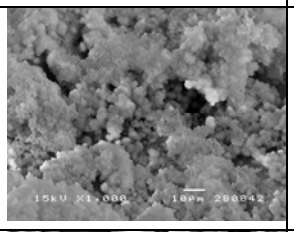
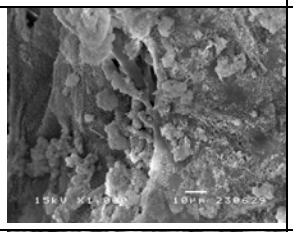
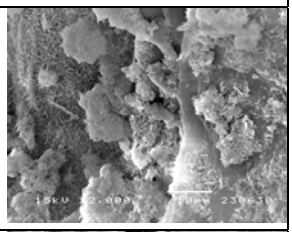
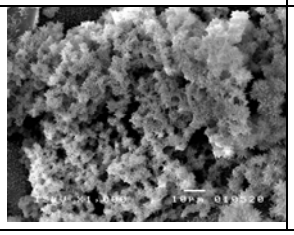
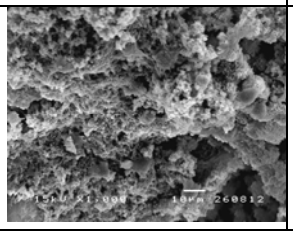
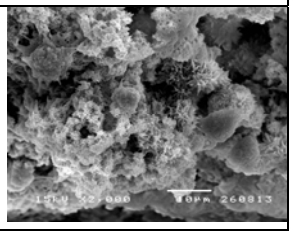
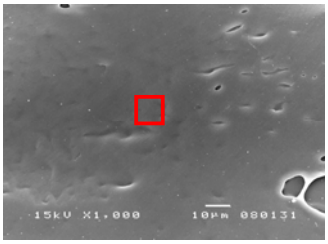
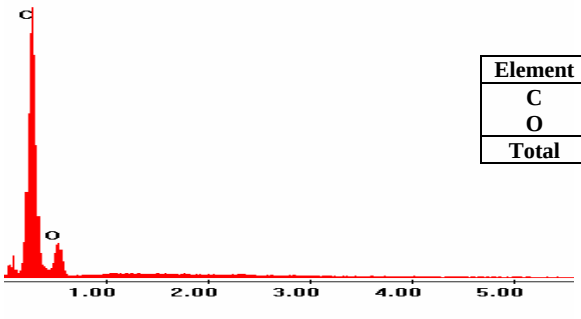
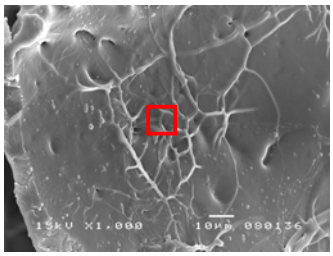
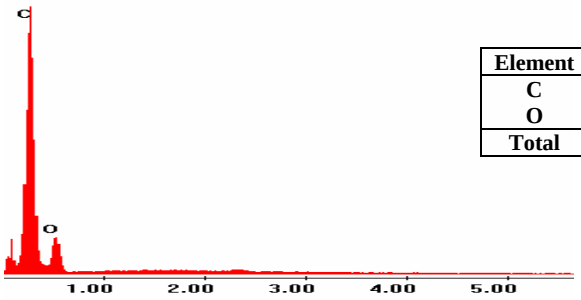
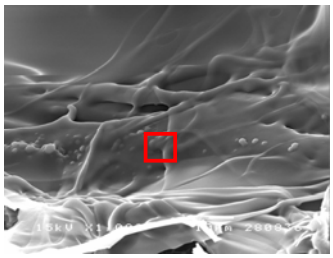
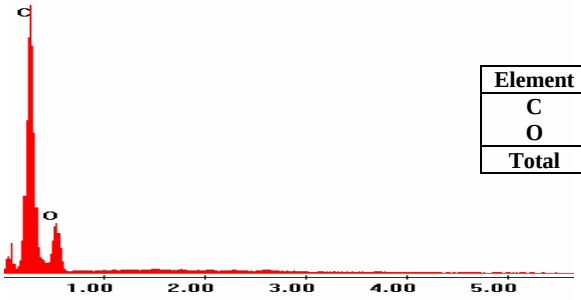
Scaffold type	Before culture	After culture in osteogenic medium	
	Scale bar : –10μm	Scale bar : –10μm	Scale bar : ——10μm
SF			
0.5%CGSF			
1.0%CGSF			
SF4			
0.5%CGSF4			
1.0%CGSF4			

Figure 4.19 Morphology of MSCs cultured on Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% (1.0%CGSF4) gelatin concentration for 29 days in osteogenic medium.

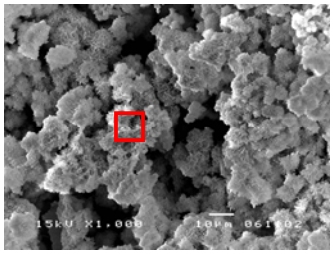
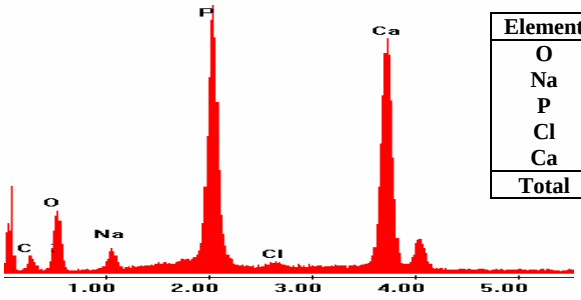
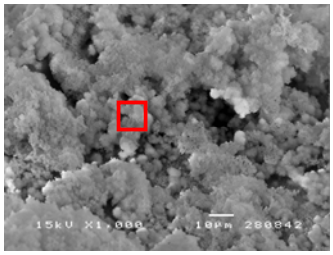
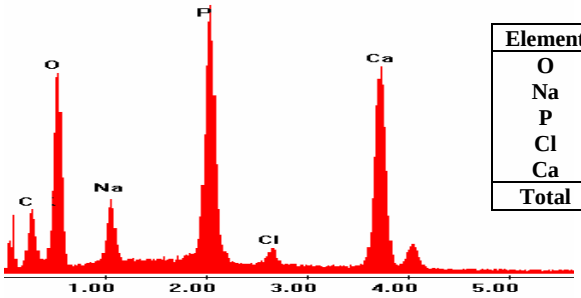
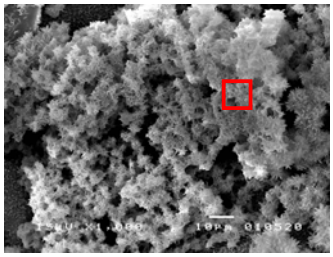
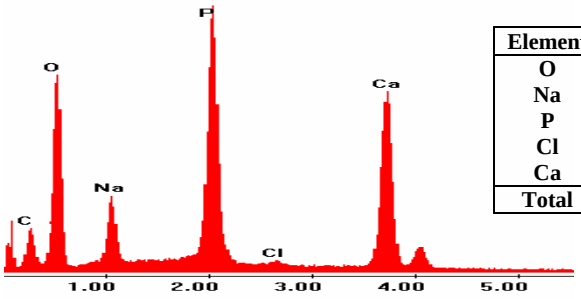
Scaffold type	Surface elements on scaffolds before cell culture
SF 	
0.5%CGSF 	
1.0%CGSF 	

Element	wt%
C	81.63
O	18.37
Total	100.00

Element	wt%
C	80.48
O	19.52
Total	100.00

Element	wt%
C	77.02
O	22.98
Total	100.00

Figure 4.20 Surface elements of Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) before cell culture, analyzed by EDX.

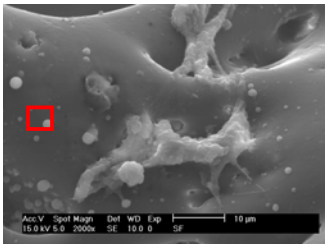
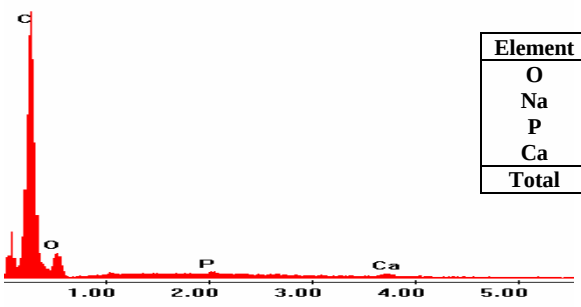
Scaffold type	Surface elements on scaffolds before cell culture
SF4 	
0.5%CGSF4 	
1.0%CGSF4 	

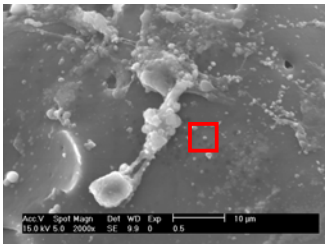
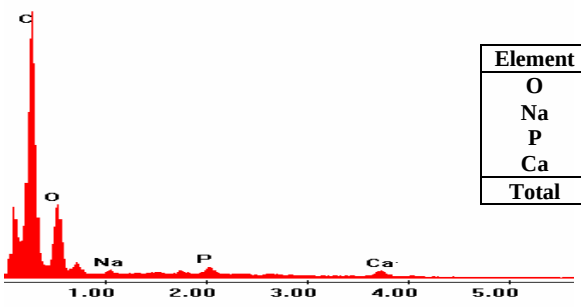
Element	wt%
O	44.50
Na	7.02
P	19.49
Cl	0.74
Ca	28.26
Total	100.00

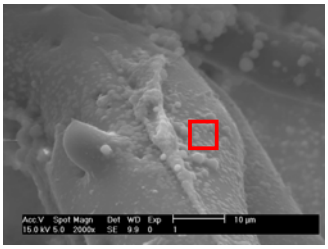
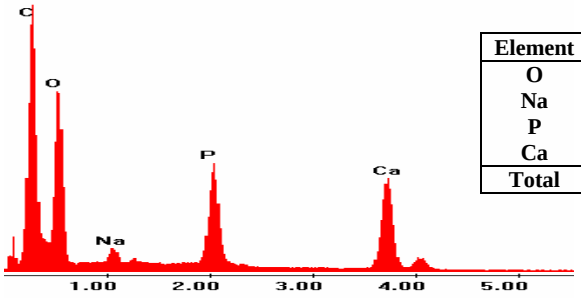
Element	wt%
O	45.01
Na	6.30
P	19.95
Cl	1.99
Ca	26.75
Total	100.00

Element	wt%
O	49.62
Na	3.00
P	20.14
Cl	0.75
Ca	26.49
Total	100.00

Figure 4.20 (continued) Surface elements of Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) before cell culture, analyzed by EDX.

<table>
<tr><th>Scaffold type</th><th>Surface elements on scaffolds after cell culture</th></tr>
<tr><td>SF
  </td><td> 

<table>
<tr><th>Element</th><th>wt%</th></tr>
<tr><td>O</td><td>93.78</td></tr>
<tr><td>Na</td><td>2.51</td></tr>
<tr><td>P</td><td>1.50</td></tr>
<tr><td>Ca</td><td>2.21</td></tr>
<tr><td>Total</td><td>100.00</td></tr>
</table></td></tr>
<tr><td>0.5%CGSF
  </td><td> 

<table>
<tr><th>Element</th><th>wt%</th></tr>
<tr><td>O</td><td>90.68</td></tr>
<tr><td>Na</td><td>2.20</td></tr>
<tr><td>P</td><td>2.76</td></tr>
<tr><td>Ca</td><td>4.36</td></tr>
<tr><td>Total</td><td>100.00</td></tr>
</table></td></tr>
<tr><td>1.0%CGSF
  </td><td> 

<table>
<tr><th>Element</th><th>wt%</th></tr>
<tr><td>O</td><td>64.73</td></tr>
<tr><td>Na</td><td>3.36</td></tr>
<tr><td>P</td><td>12.50</td></tr>
<tr><td>Ca</td><td>19.40</td></tr>
<tr><td>Total</td><td>100.00</td></tr>
</table></td></tr>
</table>

Figure 4.21 Surface elements of Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) after MSCs culture under osteogenic medium for 29 days.

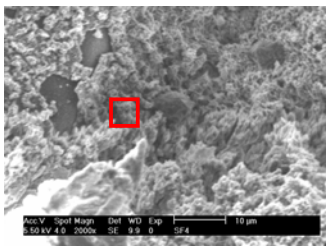
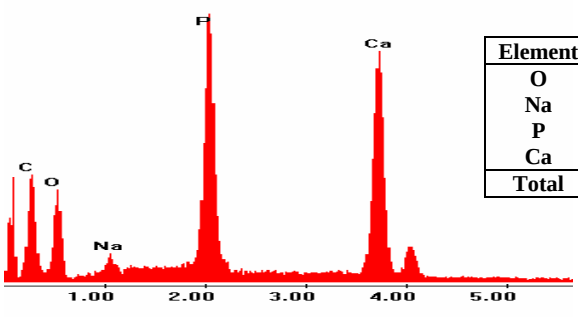
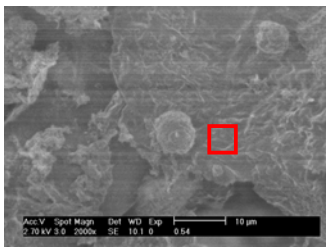
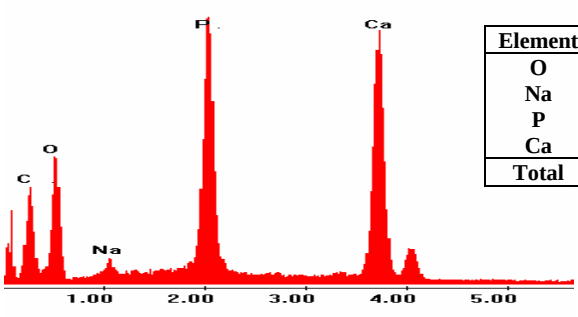
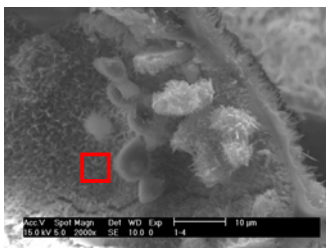
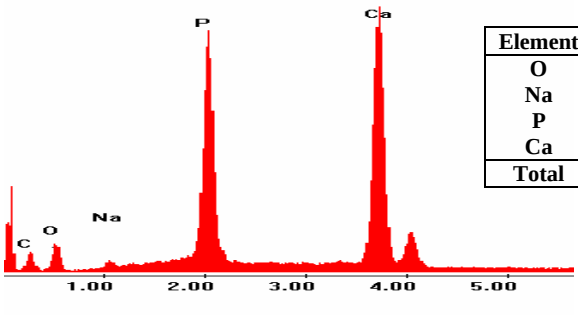
Scaffold type	Surface elements on scaffolds after cell culture
SF4 	
	Element / **wt%**: O 32.56; Na 1.60; P 26.03; Ca 39.82; Total 100.00
0.5%CGSF4 	
	Element / **wt%**: O 25.20; Na 1.48; P 27.31; Ca 46.00; Total 100.00
1.0%CGSF4 	
	Element / **wt%**: O 16.61; Na 1.13; P 29.43; Ca 52.83; Total 100.00

Figure 4.21 (continued) Surface elements of Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) after MSCs culture under osteogenic medium for 29 days.

Table 4.5 Comparison of %calcium and %phosphate on Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) before and after MSCs culture under osteogenic medium for 29 days.

Scaffold Type	**Scaffold surface Before cell culture**		**Scaffold surface after cell culture**			**Increasing in Ca and P**	
	Ca (%)	**P (%)**	**Ca (%)**	**P (%)**	**Ca:P ratio**	**Ca (%)**	**P (%)**
SF	0	0	2.21	1.50	1.47	+2.21	+1.50
0.5%CGSF	0	0	4.36	2.76	1.58	+4.36	+2.76
1.0%CGSF	0	0	19.40	12.50	1.55	+19.40	+12.50
SF4	28.26	19.49	39.82	26.03	1.53	+11.56	+6.54
0.5%CGSF4	26.75	19.95	46.00	27.31	1.68	+19.25	+7.36
1.0%CGSF4	29.49	20.14	52.83	29.43	1.79	+23.34	+9.29

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, salt-leached Thai silk fibroin scaffolds were modified by gelatin conjugation and hydroxyapatite deposition for bone tissue engineering applications. The effects of gelatin concentration and parameters of alternate soaking process on the physical and biological properties were investigated. The difference in gelatin concentration solution influenced gelatin contents incorporated into scaffolds, morphology of the scaffolds and cell proliferation on the scaffolds. The amount of gelatin incorporated into Thai silk fibroin scaffolds increased as the gelatin concentration used was increased. In addition, morphology of the scaffolds obtained from low concentration of gelatin solution (0.5wt%) showed fiber-like structure while the scaffolds obtained from high concentration of gelatin solution (1.0wt% and1.5wt%) showed more membrane-like structure.

To deposit hydroxyapatite into Thai silk fibroin-based scaffolds using alternate soaking process, various parameters including soaking time in each solution, cycles of alternate soaking and alternate soaking steps were evaluated. The amount of hydroxyapatite deposited into scaffolds increased as increasing the soaking time in each solution and the cycles of alternate soaking. The deposition of hydroxyapatite into Thai silk fibroin scaffolds and conjugated gelatin/Thai silk fibroin scaffolds resulted in less pore volume and higher compressive modulus comparing to each initial scaffolds without hydroxyapatite. The results on TGA indicated that hydroxyapatite deposited after four cycles of alternate soaking in the scaffolds were about 52%. Furthermore, both types of scaffolds soaking first in calcium chloride solution following by soaking in disodium hydrogenphosphate solution showed the suitable compressive modulus. The results on elemental analysis demonstrated that the Ca/P ratio of hydroxyapatite/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 4 cycles of alternate soaking were 1.71 and 1.51, respectively.

The results on *in vitro* cell proliferation indicated that conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration showed the highest number of proliferated bone-marrow derived mesenchymal stem cells (MSCs). In case of MSCs cultured in osteogenic medium, the results of ALP activity and calcium contents showed that conjugated gelatin/Thai silk fibroin scaffolds prepared from 1.0wt% gelatin concentration could enhance osteoconductive potential as good as the protein scaffolds with hydroxyapatite incorporation. This result revealed that hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds could serve as new alternative material for bone tissue engineering.

5.2 Recommendations

Although the effects of gelatin conjugation, and the effects of hydroxyapatite deposition on the physical and biological properties of Thai silk fibroin scaffolds have been investigated in this work, there are other interesting points which should be further considered as follows:

1. The scaffolds which showed osteoconductive potential should be investigated *in vivo* for bone tissue engineering.

2. To ensure the bone-marrow derived mesenchymal stem cells differentiation, the gene expression such as osteocalcin and osteopontin should be examined.

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APPENDICES

APPENDIX A

Elemental analysis

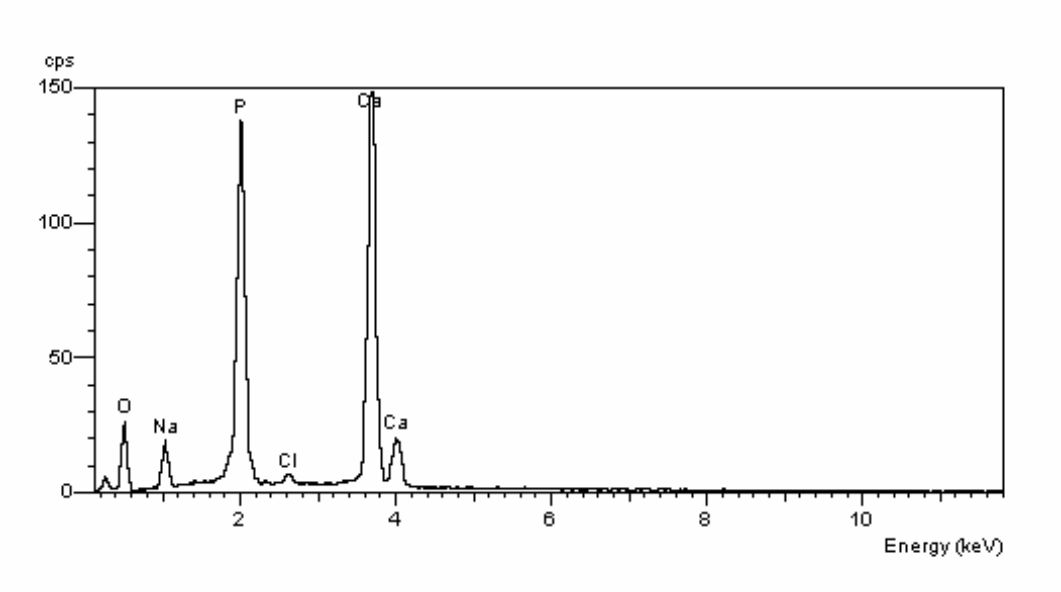


Figure A-1 Surface elements on hydroxyapatite/Thai silk fibroin scaffolds prepared from soaking first in calcium chloride solution followed by disodium hydrogenphosphate solution process.

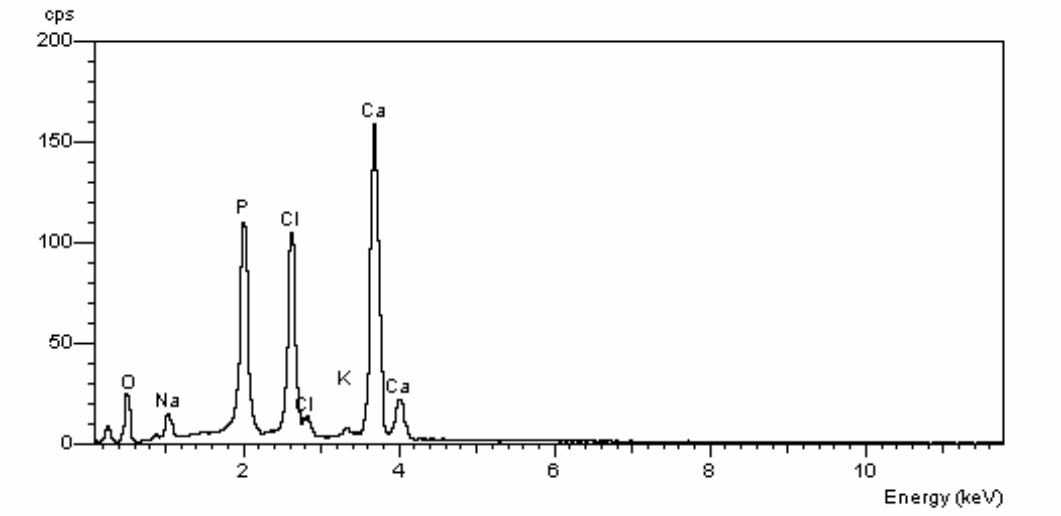


Figure A-2 Surface elements on hydroxyapatite/Thai silk fibroin scaffolds prepared from soaking first in disodium hydrogenphosphate solution followed by calcium chloride solution process.

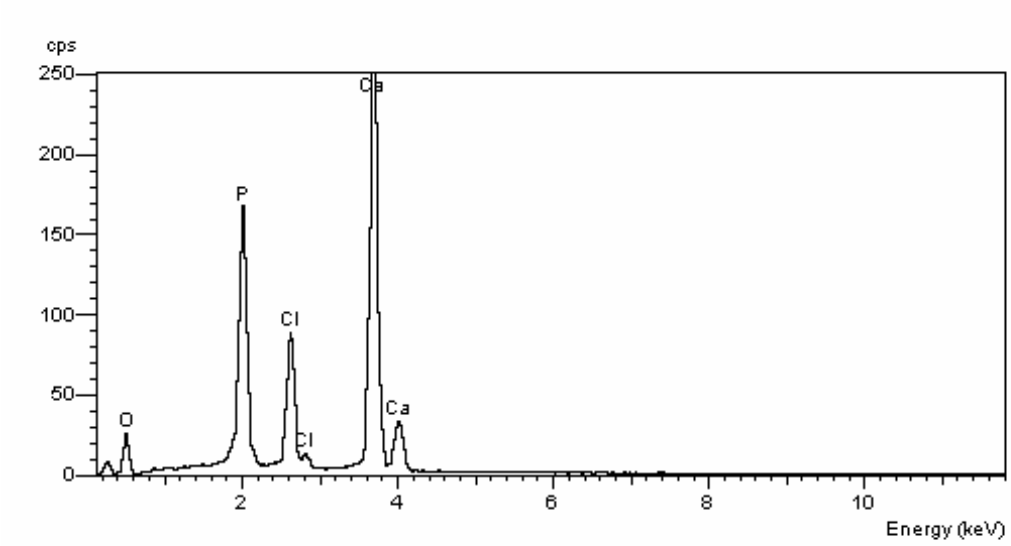


Figure A-3 Surface elements on hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from soaking first in calcium chloride solution followed by disodium hydrogenphosphate solution process.

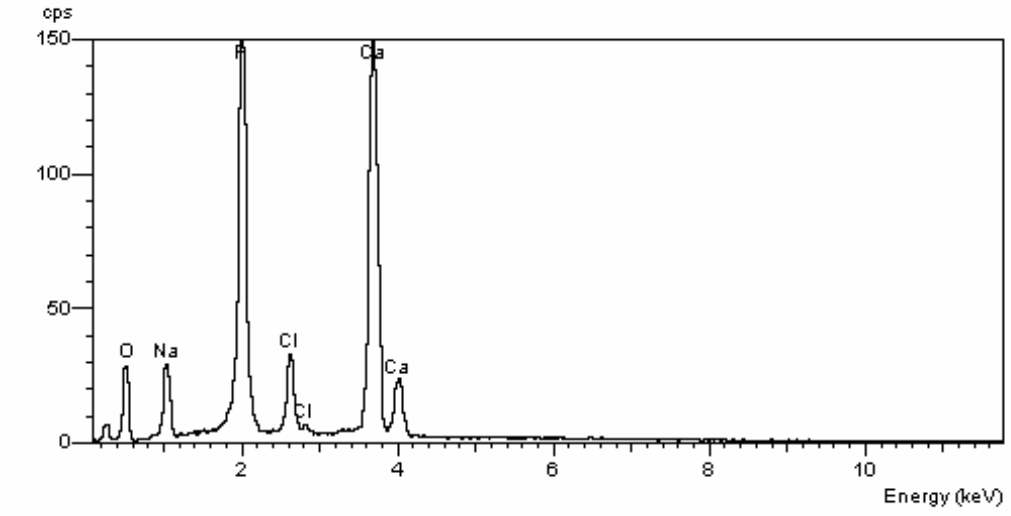


Figure A-4 Surface elements on hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from soaking first in disodium hydrogenphosphate solution followed by calcium chloride solution process.

APPENDIX B

Standard curve for DNA assay

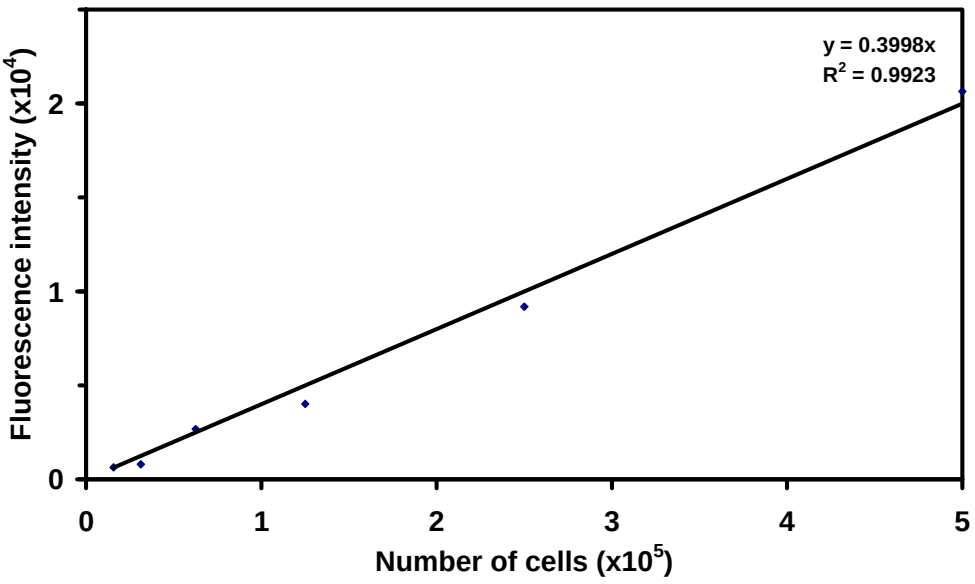


Figure B-1 Standard curve of MSCs number for DNA assay

APPENDIX C

Standard curve for *p*-nitrophenyl phosphate assay

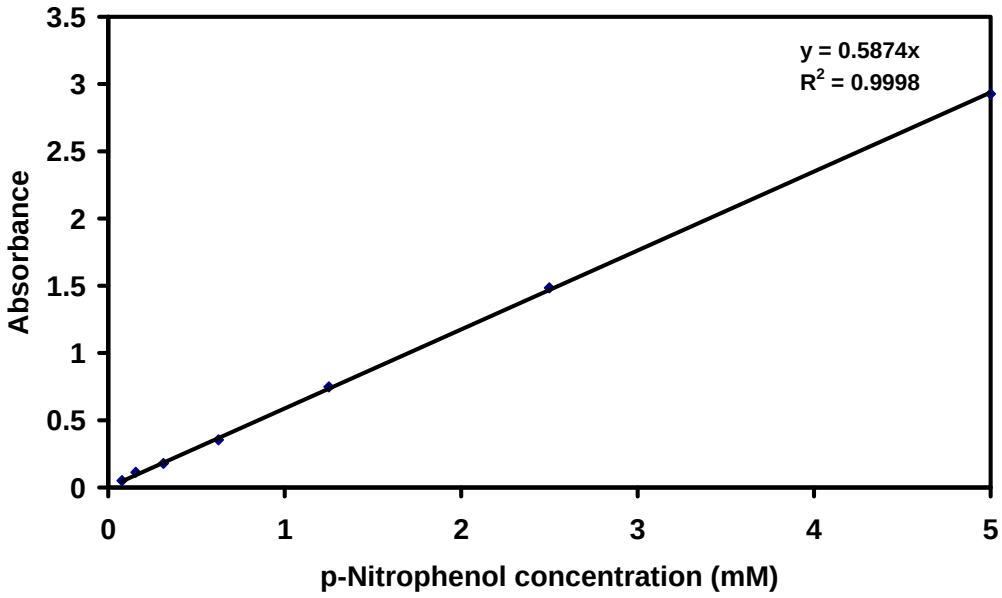


Figure C-1 Standard curve of *p*-nitrophenyl phosphate assay (ALP)

APPENDIX D

Standard curve for O-cresolphythalein assay

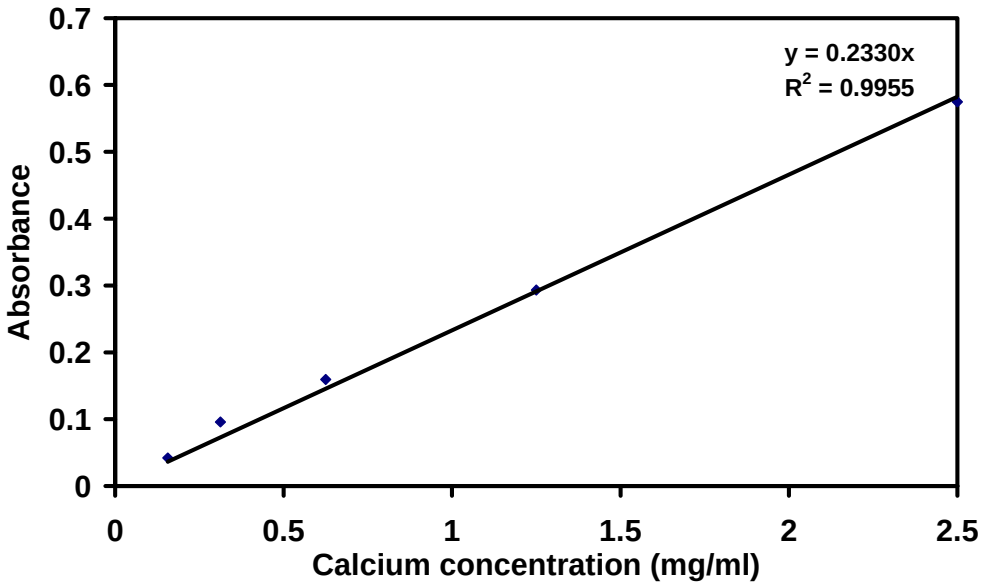
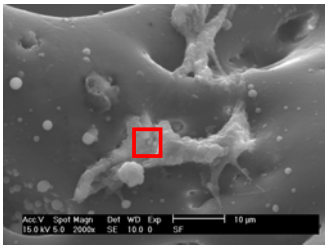
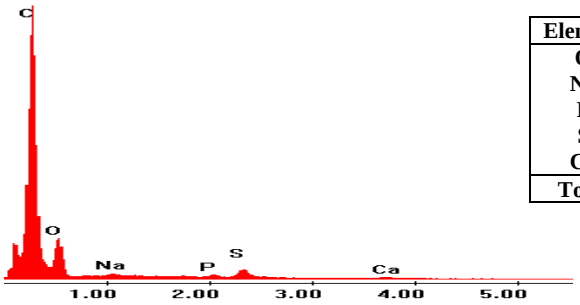
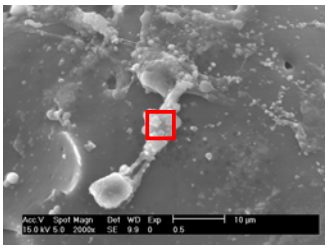
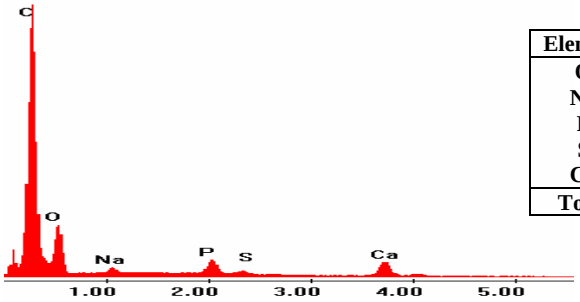
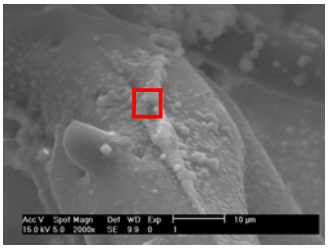
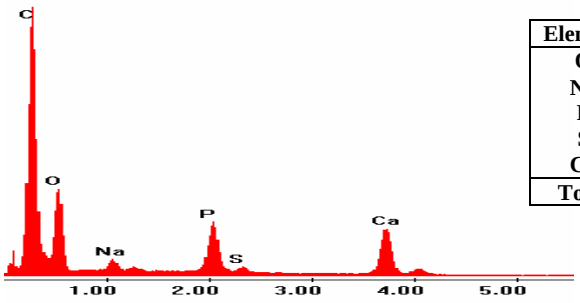


Figure D-1 Standard curve of O-cresolphythalein assay

APPENDIX E

Surface elements of cell

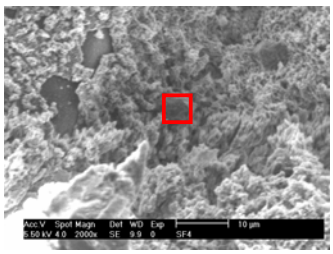
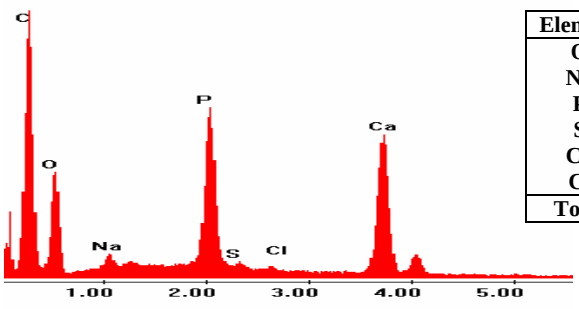
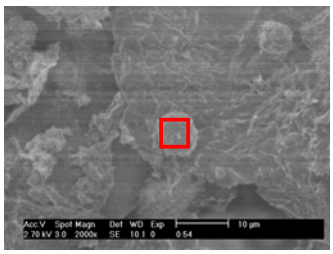
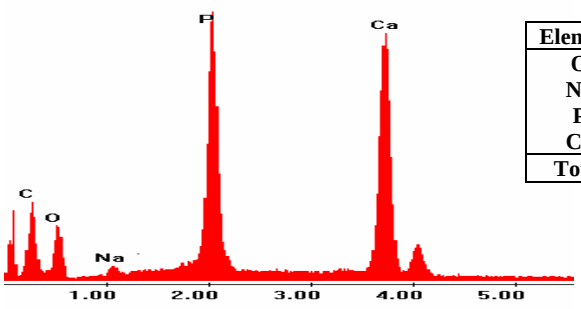
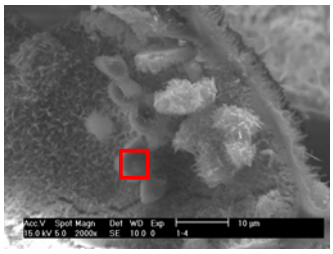
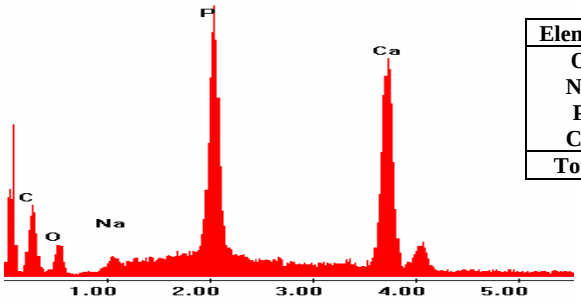
Scaffold type	Surface elements on cell
SF 	
0.5%CGSF 	
1.0%CGSF 	

Element	wt%
O	88.75
Na	3.36
P	1.07
S	5.50
Ca	1.31
Total	100.00

Element	wt%
O	74.50
Na	4.43
P	7.60
S	2.39
Ca	11.08
Total	100.00

Element	wt%
O	63.88
Na	4.19
P	11.89
S	1.42
Ca	18.62
Total	100.00

Figure E-1 Surface elements of cell on Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) after MSCs culture under osteogenic medium for 29 days.

Scaffold type	Surface elements on cell
SF4 	
0.5%CGSF4 	
1.0%CGSF4 	

Element	wt%
O	45.49
Na	2.46
P	19.97
S	0.81
Ca	30.83
Cl	0.44
Total	100.00

Element	wt%
O	23.47
Na	1.74
P	27.89
Ca	46.91
Total	100.00

Element	wt%
O	16.61
Na	1.62
P	31.16
Ca	50.16
Total	100.00

Figure E-1 (continued) Surface elements of cell on Thai silk fibroin scaffolds (SF), conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF) and 1.0wt% (1.0%CGSF) gelatin concentration, hydroxyapatite/Thai silk fibroin scaffolds (SF4), hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds prepared from 0.5wt% (0.5%CGSF4) and 1.0wt% gelatin concentration (1.0%CGSF4) after MSCs culture under osteogenic medium for 29 days.

BIOGRAPHY

Miss Shatshawan Tritanipakul was born in Bangkok, Thailand on January 2, 1984. She finished the high school education in 2002 from Saint Joseph Convent school. In 2005, she received her Bachelor Degree of Science with a major of Biotechnology from Faculty of Science, King Mongkut's Institute of Technology Ladkrabang. After the graduation, she pursued her graduate study Master of Engineering with a major of Chemical Engineering in Faculty of Engineering, Chulalongkorn University.

Some parts of this work were presented at the conferences as follows;

- The 13th International Conference on BioMedical Engineering (ICBME 2008), Suntec International Convention & Exhibition Centre, Singapore, December 3-6, 2008 (poster presentation).