CONTROLLED RELEASE OF FIBROIN REPEAT-TAGGED PROTEINS FROM SILK HYDROGEL

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การปลดปล่อยแบบควบคุมของโปรตีนที่เชื่อมกับส่วนซ้ำไฟโบรอินไหมจากไฮโดรเจล

นายจตุรงค์ พรหมสุข

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       โปรตีนชีวภาพที่มีคุณสมบัติเป็นยาอย่างเช่น Growth Factor มีบทบาทสำคัญในการแพทย์ปัจจุบัน โปรตีนชีวภาพนี้มี
ความสามารถในการกระตุ้นและซ่อมแซมเนื้อเยื่อเฉพาะจุด โดยนำ Growth factor นี้รวมกับโครงข่ายเซลล์เช่น ไฮโดรเจล จากนั้น
นำเข้าสู่บริเวณที่ต้องการซ่อมแซม อย่างไรก็ตามการแพร่กระจายของ Growth factor ออกจากบริเวณที่ต้องการหรือระยะเวลาใน
การปล่อยไม่เหมาะสมนั้น ก่อให้เกิดปัญหาต่างๆ เช่น การกระตุ้นการเกิดเนื้องอกในบริเวณต่างๆของร่างกาย หรือเกิดการอักเสบที่
ควบคุมไม่ได้ ดังนั้นวัตถุประสงค์ของงานวิจัยนี้เพื่อสร้างแบบจำลองการแพร่ของโปรตีนชีวภาพจากไฮโดเนเจลโดยใช้โปรตีนเรืองแสง
สีเขียว (GFP) เป็นตัวแทนของโปรตีนชีวภาพและงานวิจัยนี้ใช้โปรตีนไฟโบรอิน (SF) จากไหมเป็นโครงข่ายของเซลล์ไฮโดรเจล ดังนั้น
เพื่อศึกษาการปลดปล่อย GFP เป็นโปรตีนชีวภาพต้นแบบซึ่งมี SF เชื่อมติดอยู่ (SF-GFP) SF-GFP ที่สร้างขึ้นมีลำดับกรดอะมิโนใน
โปรตีน Silk fibroin ที่มีลำดับซ้ำๆ กันคือ (GAGAGS)$_n$ ลำดับกรดอะมิโนที่ซ้ำกันของโปรตีน SF เป็นส่วนที่ทำให้เกิดโครงสร้างแบบ
β-sheet ภายในโปรตีน SF การศึกษาระยะเวลาของโปรตีน SF ขึ้นรูปเป็น hydrogel พบว่า SF-GFP ส่งผลให้โปรตีน SF ขึ้นรูป
เป็น hydrogel ได้เร็วขึ้น การปลดปล่อยของ SF-GFP จาก SF hydrogel สามารถลดผลของการปลดปล่อยแบบรวดเร็วและรักษา
ระดับการปลดปล่อยอย่างต่อเนื่อง โดยเฉพาะอย่างยิ่ง SF-hydrogel ที่ประกอบด้วย 6 repeat units ของ silk นอกจากนี้ได้ศึกษา
โครงสร้างของ SF hydrogel พบว่าไฮโดรเจล ที่ผสมด้วยโปรตีนของ SF ที่มีส่วนซ้ำที่ยาวขึ้นทำให้ปริมาณของ β-sheet เพิ่มมากขึ้น
และยังส่งผลให้อัตราการย่อยสลายของไฮโดรเจลลดลงด้วย เมื่อศึกษากลไกการปลดปล่อยของ GFP ด้วยเทคนิค FTIR และ XRD
พบว่าการปลดปล่อยของ GFP จาก SF hydrogel ไม่ได้ขึ้นอยู่กับปริมาณของ β-sheet ใน hydrogel โดยตรง แต่การปลดปล่อย
GFP ขึ้นอยู่กับแรงยึดเหนี่ยวระหว่างโมเลกุลระหว่างส่วนซ้ำของ SF ที่สร้างขึ้นกับส่วนซ้ำ SF ของไฮโดรเจล ซึ่งยิ่งมีส่วนซ้ำที่ยาวขึ้นก็
จะมีแรงยึดเหนี่ยวระหว่างโมเลกุลที่แรงขึ้น จากผลการทดลองนี้ได้สร้างส่วนซ้ำของ SF ที่มี 6 ส่วนซ้ำเชื่อมติดกับ growth factor
(bFGF) เพื่อนำมาประยุกต์ใช้ในงานวิศวกรรมเนื้อเยื่อ พบว่าเพปไทด์ที่สร้างขึ้นสามารถทำงานได้เมื่อนำมาทดสอบการเพิ่มขึ้นของ
เซลล์ NIH-3T3 ที่มีความเข้มข้นตั้งแต่ 10 ng/ml ขึ้นไป และจากการศึกษาการเพิ่มขึ้นของเซลล์ NIH-3T3 ต่อการปลดปล่อยของ
bFGF จาก (GAGAGS)$_6$-bFGF พบว่าเซลล์จะมีการเพิ่มขึ้นแบบค่อยเป็นค่อยไปและยังคงมีการเพิ่มขึ้นของเซลล์อย่างต่อเนื่อง ซึ่ง
จากผลการศึกษานี้เป็นผลมาจากส่วนซ้ำของ SF ช่วยควบคุมการปลดปล่อยของ bFGF

สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อนิสิต ....
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Jaturong Promsuk : CONTROLLED RELEASE OF FIBROIN REPEAT-TAGGED PROTEINS FROM SILK HYDROGEL . Advisor: Kittikhun Wangkanont, Ph.D. Co-advisor: Peerapat Thongnuek, Ph.D.

Protein-based biologic drugs such as growth factors are playing important roles in modern medicine. These proteins can be used to stimulate local tissue repair. Growth factors are often incorporated into a scaffold, such as a hydrogel, and then put into the desired area of the body to achieve therapeutic effects. However, diffusion of these growth factors outside of the designated area or inappropriate release time can have deleterious consequences, such as inducing tumors in other areas of the body or uncontrolled inflammation. Therefore, this project aims to create a model to study diffusion of protein from hydrogel by using green fluorescent protein (GFP) as a biologics model and silk hydrogel as a scaffold to investigate the drug release behavior from the hydrogel. To investigate the bioactive release profile, GFP fused to silk repeating units (GAGAGS)$_n$-GFP was used as a model. In this study, the gelation time of the silk fibroin hydrogel containing GFP is faster than the pure silk fibroin hydrogel. The release of (GAGAGS)$_n$-GFP from silk fibroin hydrogel reduces the burst release effect and sustained release profile, especially silk fibroin hydrogel containing six repeats of SF tagged GFP. These results indicate that increasing the number of silk repeating unit(s) help sustain the model protein release from silk fibroin hydrogel. In addition, the release mechanism was investigated by FTIR and XRD method. The result showed that the release profile of GFP was not dependent on the β-sheet content of hydrogel but could be occur from hydrophobic interaction between repeating unit of peptide and repeating unit of SF hydrogel scaffold. After determining the optimal number of repeating units, the six repeating units tagged-basic fibroblast growth factor (bFGF) is produced and showed the function. The bFGF-incorporated hydrogel is examined for bFGF release and stimulated of cell proliferation in cell culture. These results indicate that six repeating units-tagged bFGF can sustain release of bFGF detected by NIH3T3 proliferation.

จุฬาลงกรณ์มหาวิทยาลัย
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Academic Year:	2020	Advisor's Signature	
		Co-advisor's Signature	

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LIST OF ABBREVIATION

A	Alanine
A_x	Absorbance at x nm
bFGF	Basic fibroblast growth factor
CMP	Collagen mimic peptide
CRS	Control drug release system
DDS	Drug delivery system
DMEM	Dulbecco's modified engle medium
DTT	Dithiothreitol
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
FTIR	Fourier transforms infrared spectroscopy
G	Glycine

GFs	Growth factor
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB medium	Luria-Bertani medium
MBP	Maltose binding protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MWCO	Molecular weight cut off
OD	Optical density
PBS	Phosphate-buffer saline
PEI	Poly(ethyleneimine)
PMSF	Phenylmethylsulfonyl fluoride
S	Serine
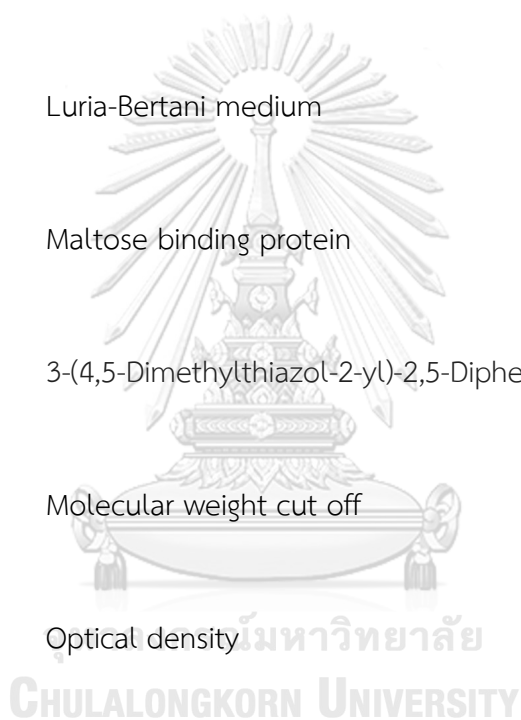
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Silk fibroin
SOC	Super optimal broth with catabolite repression
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
TEV	Tobacco etch virus
Tris	Tris-EDTA buffer
U	Enzyme unit
UP	Ultra-purified water
UV	Ultraviolet
XRD	X-ray diffraction

CHAPTER I

INTRODUCTION

1.1 Rationales and Theories

Generally in the treatment of patients, drugs are usually applied at a fixed concentration and, ideally, the drug is released in a controlled and sustained manner. In reality, drug delivery is hardly controlled because drug release rate, drugs and tissue interaction, and drug stability are difficult to handle. To solve these limitations, drug delivery system (DDS) designed to increase the efficiency of the release and maintain the proper concentration (Tibbitt et al., 2016). There are many classes of DDS. One of them is the controlled drug release system (CRS) which is engineered to deliver drugs for days to years with a predetermined release profile (Tibbitt et al., 2016). An ideals CRS has several advantages. For example, it should maintain drug concentration within the therapeutic window. A CRS should seek to improve adherence by decreasing the number of required doses that reduce the total amount of drug needed for the therapeutic effect. Finally, the CRS should be

able to deliver a drug that rapidly degraded when administered on its own. CRS

production is challenging because it requires a vehicle that can house a sufficient

quantity of therapeutic, protect the therapeutic molecule, and has a predictably

release lifetime (Mehtani et al., 2019).

Material production as a vehicle for carrying therapeutic has been studied in

synthetic polymer, chemistry, material science, medical chemistry, and conjugated

chemistry, and engineered to control the release using different material strategies

(Sung & Kim, 2020). For example, in a matrix-based system, the drug diffuses through

a network of interconnected pores. In a reservoir, the drug passes through a

membrane. In degradable DDS, the drug is released when the materials degrade

throughout. Finally, hydrogel-based DDS release drug through a constrained network

whose mesh size depends on hydration and polymer architecture (Tibbitt et al.,

2016). In the 1960s, the silicone tubes are designed for the hydrophobic lipophilic

small molecules diffuse through the device over days to months. This device

exhibited controlled compound release in the body and led to the development of

the improved CRS (Hoffman, 2008). Nowadays, polymer systems for CRS have been

extensively studied as provide the ability for prolonged delivery, low toxicity, and are

easy to manipulate and administer. In particular, synthetic polymers that are

biodegradable polymer is preferred for use as a vehicle in CRS because they can

preserve their properties for some time before they slowly dissolved into non-toxic

products that can be excreted from the body. For example, poly(ethylene-co-vinyl

acetate) were solubilized and mixed with lyophilized therapeutic protein and was

used to release therapeutic protein at desire concentration and targeting (Akl et al.,

2018). Another synthetic polymer is PEGA microspheres. This polymer is used as the

vehicle for carrying and releasing prostate cancer and endometriosis drugs (Freiberg &

Zhu, 2004). However, a synthetic polymer that are used as drug carriers are often

synthesized using organic solvents, resulting in remaining toxic residues that limits its

biocompatibility (Hamid Akash et al., 2015). On the other hand, natural polymers can

be alternative for drug carriers because they can process in milder conditions. For

example, Albumin can be used as a natural polymer vehicle because of its good

water-solubility, readily availability, biodegradability, lack of toxicity, minimal

immunogenicity, and preferential accumulation in tumor and inflamed tissues

(Elzoghby et al., 2012). It can carry drugs through two main mechanisms: albumin-drug conjugation such as a methotrexate-albumin conjugate (MTX-HAS) and drug encapsulation into albumin nanoparticles such as Abraxane. Moreover, collagen can be a carrier in the drug delivery system (Panduranga Rao, 1996). For example, there have been reported on sustained-release preparations for antibiotics such as gentamycin using collagen as a carrier. Chitosan-based drug delivery system has the potential for as use as material in CRS such as chitosan-O-isopropyl-5-O-d4T monophosphate was synthesized for the treatment of HIV infection (Hu et al., 2013). However, most natural biopolymers are hydrophilic and are rapidly degraded in an aqueous environment, resulting in short drug release periods (Hamid Akash et al., 2015).

The hydrophobic natural biopolymer can alternatively be used to extend the drug release time. Especially, fibroin protein is a hydrophobic natural biopolymer suitable for controlled drug delivery because it has amphipathic and tunable properties (Kim et al., 2014). Silk fibroin (SF) is an insoluble protein with a bulky hydrophobic domain secreted by silkworm *Bombyx mori* and can be easily purified.

SF has been used as a scaffolding material because of its highly adaptable properties, excellent biocompatibility, and mild foreign body response *in vivo.* Moreover, SF can self-assemble into mechanically robust materials that are also biodegradable and non-cytotoxic. In the past decades, SF has been widely investigated in biomedical and pharmaceutical fields because of its remarkable mechanical properties, good biocompatibility, controllable biodegradability, and low immunogenicity (Altman et al., 2003). For example, SF modified chitosan nanoparticle (SF-CSNP) has been widely used as a potential drug delivery system for the treatment of hepatic cancer and achieved improved cell response (Yang et al., 2015). In addition, SF has been widely used as a tissue engineering scaffold for the generation of blood vessels, skin, bone, ligaments, and nerve (Zhang et al., 2018).

SF, the main structural protein of silk, contains polypeptide chains with molecular weight in the range of 200-350 kDa. The primary structure of SF composes of repetitive blocks of hydrophobic heavy (H) chains and hydrophilic light (L) chains. The linkers between these chains are disulfide bonds. Hydrophilic L-fibroin comprises a small number of amino acid sequences: 14% of Alanine (A), 10% of Serine (S), and

9% of Glycine (G), while hydrophobic H-fibroin consists of 46% of Glycine (G), 30.0% of Alanine (A), 12.1% Serine (S), 5.3% of Tyrosine (Y), and 1.8% of Valine (V). Hydrophobic H-fibroin chains consist of repetitive hydrophobic domains interspersed between non-repetitive hydrophilic domains. The amino acid of repetitive hydrophobic domains of H-fibroin fold and bond together via hydrogen bonds, Van der Waals forces, and hydrophobic interactions to form an anti-parallel β-sheet crystalline structure. These crystalline domains are highly organized at the micro-level and act as crosslinking points in the less ordered, poorly oriented amorphous matrix, which comprises random coils, β-turns, and α-helix structures and is formed of non-repetitive domains. The strong β-sheet interactions, high degree of order, and high fibroin network and thus, confer excellent mechanical strength to the silk network. The conformation and crystallinity of SF can be adjusted to control its mechanical properties and degradation rate to make the silk fibroin control release system (Qi et al., 2017). However, the conventional SF hydrogel does not process a linear and prolonged drug release profile. For example, SF hydrogel release diclofenac sodium into PBS buffer in a nonlinear manner with a short effective

lifetime. Hence in these systems, drugs are allowed to be released by both diffusion

and degradation mechanisms, resulting in a fluctuated release profile (de Moraes et

al., 2015).

One approach to achieve an extended-release profile is to tag the drug with SF-

derived peptide that can interact with the SF hydrogel to reduce drug diffusion. Many

researchers have tried to synthesize protein repeating units that mimic the known

amino acid sequence in natural protein. For example, Collagen mimetic peptide or

CMPs that mimic the natural collagen has synthesized $(Gly-X-Y)_7$-tagged growth

factor. CMPs can bind strongly to natural collagen in wound tissue and providing the

option of a long-term attachment or its sustained release over a while. So, the CMPs-

tagged effector molecule has an advantage for delivery of therapeutic small

molecules, peptides, and proteins, and could be useful for wound healing in burn

patients or slow-healing wounds in diabetic patients (Chattopadhyay et al., 2012).

The recombinant spider silk protein eADF4 (C16) is an engineered protein that mimics

the know amino acid sequence of the natural silk protein eAD4 from the European

garden spider *Araneus diadematus*. It comprises of 16 repeats of the amino acid

sequences: GSSAAAAAAAAGPGGYGPENQGPSGPGGYGPGGP. These recombinant spider

silk proteins can be prepared into various forms and it has been shown that spider

silk is a potential candidate for a sustained drug delivery system (Spiess et al., 2010).

For example, the fusion protein GFP-eADF(C16) could self-assemble into a hydrogel

form and it retains the fluorescent property of GFP. From this report, it can be

implied that mimicking the amino acid sequences of natural spider silk to engineer

the recombinant spider silk protein could lead to an advance in CRS (Humenik et al.,

2018). Similar to spider silk, the primary structure of H-fibroin of silk has highly

repetitive amino acid units (GAGAGS)n that lead to the formation of secondary

structure β-sheet. However, the development of SF repeating units as a CRS has not

yet been studied before.

1.2 Objective of this dissertation

- To engineer SF repeating unit(s) coupled to other specific protein, green

  fluorescent protein (GFP), as a drug release model.

- To investigate the effect of SF repeating unit(s) on gelation of SF hydrogel and release profile of GFP model drug from SF hydrogel by degradation mechanism.

- To characterize the structure of SF hydrogel in complex with recombinant SF-tagged GFP and apply this modified SF hydrogel as a sustained drug release carriers.

1.3 Expected beneficial outcome from the dissertation

The SF hydrogel assembled with SF repeating unit(s) fused to bioactive molecules such as growth factors is applied and used in control drug release systems.

a

H_3N — (GAGAGS)$_n$ — GFP — COOH

n = 0,1,2,3,4,5,6,7,...

b

≡ = Repeat unit(s) of SF (β-sheet crystalline domain)

∿ = Amorphous domain

● = GFP protein

─()$_n$─● = (SR)$_n$-GFP peptide

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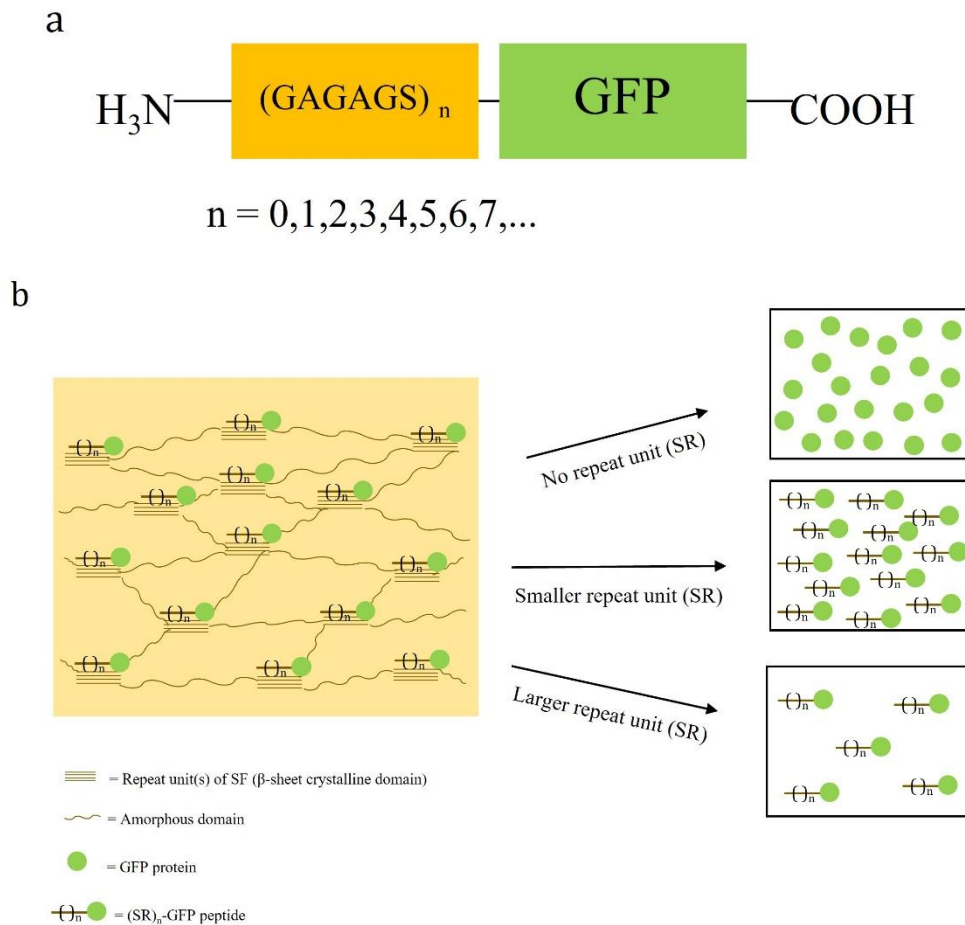
Figure 1: Illustration concept of control bioactive release of SF mimetic peptide from SF hydrogel.

a). schematics of the SF mimetic peptide tagged bioactive molecule. b). illustration of possible interaction between the SF mimic peptide and repeats unit of SF hydrogel in control release.

CHARTER II

LITERATURE

2.1 Control drug release system

Control drug release system (CRS) is the one important class of drug delivery system (DDS) that improved drug delivery, drug bioavailability, retain effective of drug concentration, and eliminate side effect when compared with traditional medicine. CRS production is carefully designed to allow the adequate incorporate therapeutics, protect the therapeutic damage *in vivo*, maintain activity, control release the therapeutic throughout the period, and biocompatible and degrade with non-toxic product (Tibbitt et al., 2016). Recently, the therapeutic protein is widely used in pharmaceutical and medical treatment but protein drug is rapidly digested before reaching the target site. To overcome this limitation, many materials production for CRS is designed in terms of polymers that are suitable for protecting therapeutic proteins from such extreme conditions. Many of synthetic polymers have been

intensively used for efficient delivery of protein and peptide drugs (Table 1), but

protein drug is rapidly digested before target site (Fenton et al., 2018).

Table 1: Synthetic polymer in therapeutic protein drugs delivery system.

Synthetic Polymer	Function
PEG	PEGylation is PEG conjugated with therapeutic protein. This polymer prevents enzymatic degradation and protein immunogenicity, prolonging in body and improving the stability, and maintain pharmacokinetics and therapeutic activity.
PLGA	PLGA is biodegraded by hydrolysis of the ester linkage into non-toxic small molecule monomers and caused a non-linear and dose-dependent profile. In addition, PLGA can be copolymerizing with other polymer for improve the efficiency delivery of therapeutic protein and peptides.
PNiPAAM	PNiPAAM has a great potential for delivery of therapeutic protein and peptides but this polymer is limited because it is non-biodegradable and activated the platelets in blood.
PF127	PF127 is made up from PEO and PPO polymer. PF127 is the biomaterial for delivery of therapeutic protein, and it has shown to be non-irritant and cytocompatibility with various cell type

However, a synthetic polymer that is used as drug carriers is often

synthesized using organic solvents, resulting in remaining toxic residues that limit its

biocompatibility. On the other hand, natural polymers can be alternative for drug

carriers because they can be processed in milder conditions (Table 2).

Table 2: Natural polymer in therapeutic protein drugs delivery system.

Natural Polymer	Function
Chitosan	Chitosan is composed of D-glucosamine and acetyl-D-glucosamine that contain amino and hydroxyl group for functionalization. Chitosan is known to promote the absorption of large molecular weight therapeutic protein through intestinal epithelial mucosa. For example, chitosan has been used to enhance the absorption of insulin. Therefore, this system can be used for drug delivery.
Alginate	Alginate has been used as a component of carrier system for efficient delivery of therapeutic protein. Alginate alone or with other copolymer have been used encapsulation of therapeutic protein. This high degree of flexibility of alginate help deliver the therapeutic protein over a period ranging from minutes to months.
Gelatin	Gelatin is a protein-natured biopolymer that has thermoreversible properties. Gelatin allows easy modification on the amino acid level and also biocompatible and biodegradable. The thermogelation behavior of gelatin was improved close to body temperature by combining gelatin with therapeutic proteins. Due to its versatile characteristics, it is widely used for delivery of therapeutic molecules via targeted drug delivery system.
	Collagen is the most abundant protein in the living organism. Collagen has been efficient result in sustained release of human

Collagen	growth hormone (hGH) resulting in better wound healing with a single administration of hGH

However, most natural biopolymers are hydrophilic and are rapidly degraded in an aqueous environment, resulting in a short drug release period.

2.2 Natural polymer-based CRS

2.2.1 Silk

Silk is the natural polymer or biopolymer produced from insects in arthropod phylum such as silkworms, spiders, bees. Silk is a popular material for use in medication processes like tissue engineering and drug delivery system. These silks that are used for a biomaterial in medical application is mulberry silkworms "*Bombyx mori*" (Porter & Vollrath, 2009)

Silk fiber from silkworm "*B. mori*" is the protein-polymer that is synthesized from two glands and keep in the lumen before secretion spinning fibers. At the molecular level, natural silkworm silk comprises two majors of structural proteins are fibroin and sericin (Table 3). Fibroin is the main component of silk, while

sericin is the outer glue-like coating. Silk fibers have two SF filament coated sericin.

SF filaments are assembled from 3-5 nm in diameter of nanofibrils. These nanofibrils

interact strongly with each other and assemble into larger fibrils are 20-200 nm in

diameter to form microfibrils. The strong friction between the nanofibrils is the main

reason for the strong interaction and cause the excellent mechanical strength of silk

fiber.

Table 3: Silk fiber components

Components	Percentage (%)	Reference
Fibroin	72-81	(Inoue et al., 2000)
Sericin	19-28	
Fat/wax	0.8-1	
Color/ash	1-1.4	
Summary	100	

Silk fibroin (SF) is the hydrophobic protein and major structure of silk. SF

provides mechanical strength, environmental stability, biocompatibility, and

morphologic flexibility. SF contains polypeptide chains with a molecular weight of

around 200-350 kDa. The primary structure of SF composes of a repetitive block of

hydrophobic heavy chains (H-fibroin) and hydrophilic light chains (L-chain). The

linkers between these chains are disulfide bonds. The structure of SF protein was

shown in Fig 1. In addition, glycoprotein P25 is present in the structure of SF that

provides non-covalently liked to the H and L chains. The molecular ratio of H-fibroin,

L-fibroin, and P25 is 6:6:1. The amino acid sequence of hydrophilic L-fibroin

comprises a small number of amino acid sequences, while hydrophobic H-fibroin has

many amino acid sequences that show in table 4.
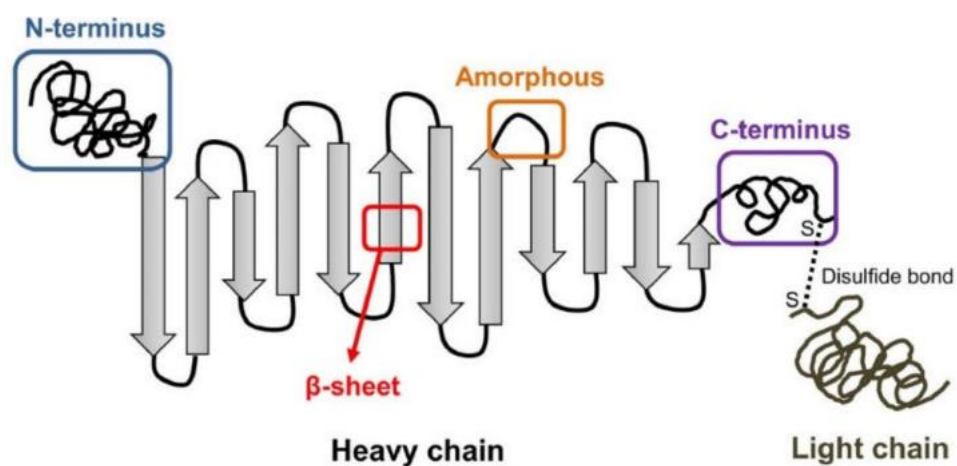
Figure 2: SF structure illustration including heavy chain and light chain (Zuluaga-Vélez et al., 2019).

Table 4: Amino acid component in H-fibroin and L-fibroin of silk fibroin.

Amino acids	mol% of amino acid residues	
	H-chain	Light-chain
Glycine	49.4	10.0
Alanine	29.8	16.9
Serine	29.8	7.9
Tyrosine	4.6	3.4
Valine	2.0	7.4
Aspartic acid	0.65	15.4
Glutamic acid	0.70	8.4
Threonine	0.45	2.8
Phenylalanine	0.39	2.7
Methionine	-	0.37
Isoleucine	0.14	7.3
Leucine	0.09	7.2
Proline	0.31	3.0
Arginine	0.18	3.8
Histidine	0.09	1.6
Lysine	0.06	1.5

From the amino acid sequence in H-fibroin, the amino acid sequence is a

repeat unit block and can be described as (-Gly-Ala-Gly-Ala-Gly-Ser-)n. The repetitive

hydrophobic amino acid domains of H-fibroin fold and bond together via hydrogen

bonds, Van der Waals forces, and hydrophobic interaction, to form an anti-parallel β-

sheet crystalline structure. These crystalline domains are highly organized at a

microscopic level and act as crosslinking points in the less ordered, poorly oriented

amorphous matrix, which comprise random coils, β-turns, and/or α-helix structures

and is formed of a non-repetitive domain that causes to finish of structure

organization. The secondary structures of silk could be classified into three crystalline

forms: silk I, silk II, and silk III. Silk I is a liquid state that is a metastable form of SF

stored in the gland of silkworms and the major structure contains α-helix and even

random coil secondary structure. Conversely, silk II is a solid-state that forms after

spinning and has a β-sheet crystalline secondary structure. Lastly, silk III is a solid

form of SF that has trifold helical chain conformation and is found at the air/water

interface. During silkworm spinning, the conformation of silk changes from a dissolved

solution of silk I to the solid form of silk II that are highly ordered structure to form

of silk fibers (Nguyen et al., 2019). To investigate the secondary of SF protein, Fourier

transforms Infrared Spectrophotometry (FTIR) was used to identify the structure of

SF. The SF structure revealed two different structure models: silk I is known to be

rich in α-helical structure occur at 1650-1658, 1545, and 1240 cm^{-1} respectively; silk II

of SF structure is known to be rich in β-sheet and had infrared absorption peaks in

the amide I, II and III at 1625-1640, 1515-1525, and 1240 cm^{-1} respectively; and those

of random coils occur at 1640-1648, 1535-1545 and 1235 cm^{-1} respectively. In addition, X-ray diffraction (XRD) is the other method for characterized the structure of SF in terms of the crystalline region in SF structure. XRD pattern peak of SF protein appears at 12.2°, 19.7°, 24.7°, and 28.2° for silk I and at 9.1°, 18.9°, and 20.7° for silk II (Wang & Zhang, 2013).

2.2.2 Silk fibroin for control drug delivery system

From the amphipathic structure of SF, the crystalline domain in SF provides excellent properties such as control drug release and degradation profile. There are various research to used SF as a biomaterial for control drug release (Tomeh et al., 2019). There has been much research to produce the SF biomaterial in various format for control and sustain drug or therapeutic agent release that showed in table 5.

Table 5: The SF biomaterial format for control drug release system.

Formulation	Therapeutic/drug	Outcome	Ref.
Disk	HIV inhibitors	Disk format of SF can stabilize microbial contamination during storage. SF disk can deliver in local route and sustained HIV inhibitor over 4 weeks.	(Zhang et al., 2017)
Films	Theophyllin	This format can used in transdermal delivery and sustained release up to a week.	(Rujiravanit et al., 2003)
	Diclofenac Sodium	SF films can cross-link with chitosan and control release over 10 days	
	Amoxicillin	SF films can be designed for pH-dependent release (pH2 > pH 5.5 > pH 7.2) over 10 hours.	
	Nerve growth factor (NGF)	Designed to be used as a nerve conduit in peripheral nerve defect. NGF release for over 3 week and conduct PC12 cells proliferation and maturation.	(Uebersax et al., 2007)
Coating	Rhodamine B	Sustained release up to 40 days with layer-by-layer silk coating	(Wang et al., 2007)
	Heparin	Multi-layer silk coating for vascular stent	(Wang et al., 2008)
	Epirubicin Hydrochloride	Layer-by-layer heparin-silk nanofilms. This system can control epirubicin hydrochloride drug release up to 7 days.	(Choi et al., 2018)
	Theophyllin	Silk used as the film coating material on a drug tablet. Drug release was sustained following zero order kinetics	(Bayraktar et al., 2005)
Forms	Vincristine	Silk forms can use in intratumoral implantation that deceased tumor growth	(J. M. Coburn et al., 2017)

		by sustained release up to 48 days.	
Microneedles	Tetracycline	This format is mild drug encapsulation method and has activity of 10-fold reduction in bacterial density.	(Tsioris et al., 2012)
	Vaccination against: Influenza	Vaccine coated on silk microneedles and showed in vivo immune response over 28 days.	(Stinson et al., 2017)
Reservoirs	Anastrozole	Silk rod reservoirs can used as implant systems for cancer therapy. This system showed sustain release up to 91 days following zero order kinetics.	(Yucel et al., 2014)
	Cisplatin	Crisplatin release over 30 days from silk reservoirs. This format can allow high drug loading and can used in intatumoral application.	(Yavuz et al., 2019)

2.2.3 Silk fibroin hydrogel for control drug release

Hydrogels are three-dimension (3D) macromolecular polymeric chains that

can be easily molded in any form, shape, and size. There is a particularly appealing

type of drug delivery system and has been used in many-branched of medicine,

including tissue engineering, cardiology, oncology, immunology, wound healing, and

pain management. Generally, hydrogels are composed of a large amount of water

and a cross-linked polymer network. The high water content provides physical

similarity to tissue and can give the hydrogel excellent biocompatibility and easily

encapsulate hydrophilic drug (Galateanu et al., 2019).

Silk materials can be fabricated into various formats such as films, particles,

and gel. Interestingly, hydrogel format has specifically aqueous-based platforms as

their properties lend unity for a diversity of medical applications. Hydrogel-based

silks have been thoroughly studied for potential biomedical applications due to their

mechanical properties, biocompatibility, controllable degradation rates, and self-

assembly into β-sheet networks.

Normally, SF hydrogel is an excellent choice for the delivery of small drug

molecules that is stabilized drug efficiency. Typically, after incorporating them into

the SF network, therapeutic agents are maintained, transported to the target sites,

and released in a controlled manner. For example, SF hydrogel incorporated with an

antibiotic such as penicillin, ampicillin, cefazolin, rifampicin, erythromycin, and

tetracycline demonstrated rapidly release in 1-5 days, but SF hydrogel preserved

antibody efficacy through *in vivo* and *in vitro* bacterial growth (Pritchard et al., 2013).

Fang et al. investigated the rapid release of buprenorphine from SF hydrogel via hydrogel mesh size and SF concentration (Fang et al., 2006). In addition, Seib et al. used an SF-based hydrogel to deliver doxorubicin (DOX) for the localized treatment of primary breast cancer. The SF-DOX hydrogel could be normally released from hydrogel and exhibit highly tumor regression response and reduced metastatic spread (J. Coburn et al., 2017). In addition, not only the delivery of small molecule drugs but also the delivery of biological molecules such as proteins, growth factors, peptides, and gene is also an important area of biomedical application. For example, In 2011 reported monoclonal antibody that could be released and maintained from SF hydrogel over an extended period by the high density of the crystalline network of hydrogel (Guziewicz et al., 2011). Uebersax et al. used SF-base hydrogel blended with nerve growth factor (NGF) for control release of growth factor in tissue engineering. Afterward, it was demonstrated that the proliferation and differentiation of PC12 cells that caused by sustained release of NGF from hydrogel (Uebersax et al., 2007) but this process occurs rapidly bioactive release in uncontrolled fashion as shown in Fig 2. To improve these limitation, SF-based hydrogel mixed with other

polymeric molecules was used in control bioactive release to improve release efficacy. For example, SF/polyacrylamide (PAAm) is an example of an SF-based protein delivery system for the delivery of FITC-labelled insulin (Mandal et al., 2009). The release profile of insulin of this hydrogel was controlled and sustained release by increasing additional polymer. In addition, the vascular endothelial growth factor (VEGF) was used as a cytokine model and was non-covalently encapsulated into composite gels before crosslinking it in the presence of polyethylene glycol diacrylate. The additional of 0.3% thiol-modified heparin to the release medium led to the increase in the present release of VEGF from 25% to 75% over 42 days (Elia et al., 2013). In 2015, composite hydrogels were prepared by blending SF with the copolymer. Aspirin and indomethacin were incorporated into SF/copolymer hydrogels as two model drugs with different water solubility. The hydrophilic and hydrophobic drug release was showed differently release profiles upon different of interaction between drug and copolymer (Zhong et al., 2015).
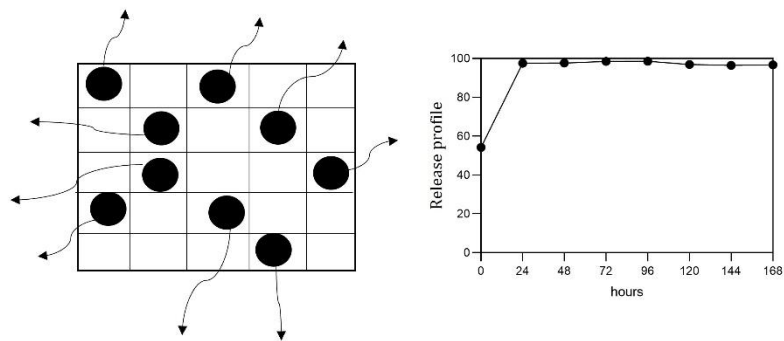
Figure 3: Burst release effect of bioactive molecule from SF hydrogel matrix

2.3 Biomimetic peptide for control drug release

Biomimetic peptides are synthetic compounds that are identical to amino acid sequences synthesized by chemical or biological processes (Vincent, 2014). For example, Collagen mimetic peptide or CMPs that mimic the natural collagen has synthesized (Gly-X-Y)$_7$-tagged growth factor. CMPs can bind strongly to natural collagen in wound tissue and providing the option of a long-term attachment or its sustained release over a while. So, the CMPs-tagged effector molecule has an advantage for delivery of therapeutic small molecules, peptides, and proteins, and could be useful for wound healing in burn patients or slow-healing wounds in diabetic patients (Chattopadhyay et al., 2012). In the case of silk, much research was on synthesized recombinant spider silk mimic peptides because it is particularly

difficult to domesticate. Most recombinant spider silk proteins are based on *Nephila*

clavipes refer to their spidroins as MaSp1 and MaSp2 depending on proline amino

acid content. This recombinant was showed delivery of DNA plasmid to a tumor

target site (Numata et al., 2012). Moreover, the recombinant spider silk protein eADF4

(C16) is an engineered protein that mimics the know amino acid sequence of the

natural silk protein eAD4 from European garden spider *Araneus diadematus*. It

comprises of 16 repeats of the amino acid sequences:

GSSAAAAAAAAGPGGYGPENQGPSGPGGYGPGGP. These recombinant spider silk proteins

can be prepared into various forms, and it has been shown that spider silk is a

potential candidate for a sustained drug delivery system. (Lammel et al., 2011). In

addition, the fusion protein GFP-eADF(C16) could self-assemble into a hydrogel form

and it retains the fluorescent property of GFP. From this report, it can be implied

that mimicking the amino acid sequences of natural spider silk to engineer the

recombinant spider silk protein could lead to an advance in the drug delivery system

(Humenik et al., 2018).

Accordingly, the structure in SF of silk from *Bombyx mori* that has a repeating block in a hydrophobic domain can control its mechanical properties and degradation rate to make the SF control release system. For example, Silk-elastin-like protein polymers (SELPs) are block copolymers consisting of an amino acid repeat (GAGAGS) of SF from *B. mori* silk and mammalian elastin (GVGVP). SELPs have been examined for controlled release of markers and bioactive agents from low molecular weight to high molecular weight controlling by a concentration of polymer and development of structure of this polymer (Dinerman et al., 2002). In addition, Gene delivery from SELPs has also been explored by examining the character of DNA plasmid release but the route of gene transfer often lacks the efficiency (Megeed et al., 2002).

2.4 Control release of biosignalling on tissue engineering application

Tissue engineering is a new area of biomedical technology and methodology to assist and accelerate the regeneration of damaged tissue based on the natural healing of patients themselves. Biomaterial technology plays an important role in the creation of cell environments such as scaffold and drug delivery systems of signaling

molecules that have been investigated to enhance proliferation and differentiation of

cell potential for tissue engineering.

Basically, body tissue is composed of cells, and the surrounding environment

that includes extracellular matrix (ECM) for cell proliferation and differentiation as the

living places of cells and signaling molecules. There is some case where tissue

regeneration is involved by single or combination of cell and the environment

surrounding. To the end, biomaterial plays a key role in designing and creating

substitutes for ECM and the drug delivery system (DDS) of signaling molecules to

enhance their biological application. As the biomaterial, various synthetic and natural

materials, such as polymers, ceramics, and metals or their composites, have been

investigated and used in different manners. Among them, biodegradable biomaterials

are an example in table 6 (Tabata, 2003).

Table 6: Biodegradable polymers used for tissue engineering of cell scaffold and biosignaling molecules release.

Synthetic polymer	Natural polymer
Poly(L-lactic acid) (PLLA)	Collagen
Poly(glycolic acid) (PGA)	Gelatin
Poly(e-caprolactone) (PCA)	Fibrin
Copoly(LL-GA)	Hyaluronic acid
Copoly(LLCA)	Alginate
Copoly(LLA-ethylene glycol (EG))	Chitosan
Copoly(fumarate-EG)	Chitin

Although the growth factors are playing important role in tissue regeneration, the directly inject of growth factor into site effect is not effective. The growth factors are rapidly diffused from the injected site and rapidly degrade with local enzymatic. To overcome this limitation, the growth factors are incorporated into biomaterial for control growth factor release in appropriated manner but this process still has uncontrolled release profile as shown in figure 3.
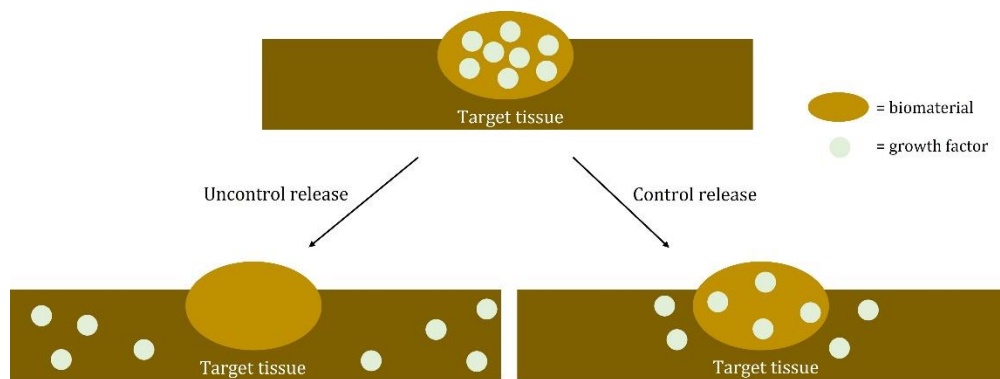
Figure 4: Growth factor release from biomaterial at target site in tissue engineering with uncontrol release and control release profile

2.4.1 Biosinalling in tissue engineering

Growth factors (GFs) are a type of biosignaling molecule that are powerful

therapeutic agents for tissue regeneration. GFs are soluble proteins secreted by cells

that can regulate a wide range of cellular behaviors, such as proliferation, migration,

and differentiation, by binding to specific transmembrane receptors on target cells

(Tabata, 2003). Some of the widely studied GFs in tissue regeneration and repair are

involved in angiogenesis and osteogenesis. These include vascular endothelial growth

factors (VEGFs) (Brandi & Collin-Osdoby, 2006), fibroblast growth factor (FGF) (Martino

et al., 2015), and placental growth factors (PIGFs) which are involved in angiogenesis

and play a crucial role in the repair of most tissue, insulin-like growth factors (IGFs),

transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs) (Kirker-Head, 2000) which are the most extensively used osteogenic proteins for inducing new bone formation in bone defects and ectopic sites.

2.4.1.1 Basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor (bFGF) is one type of fibroblast growth factor and shows a widely of biological activity. bFGF has 9.6 isoelectric points (pI) and was firstly identified by its ability to cause the proliferation and phenotypic transformation of NIH-3T3 fibroblast cell (Gospodarowicz, 1974). Most of the biological studies with fibroblast growth factors have been done with basic form (bFGF). bFGF has both acute and long-term effects on the morphology and growth pattern of responsive cells. Human skin fibroblasts maintained with bFGF in serum-supplemented medium become extremely elongated with slender projections. In addition, bFGF is a potent mitogen for mesoderm-derived cells. When tested on vascular endothelial cells derived from either large vessels or capillaries, it triggers cell proliferation at a concentration as low as 2 pg/ml. This concentration is 20 to 60-fold lower than those required to see comparable effects on cell growth or the

initiation of DNA synthesis with TGFB, EGF, or PDGF. bFGF stabilizes the phenotypic

expression of cultured cells. This factor has made possible the long term culturing of

the cell types that otherwise would lose their normal phenotypes in culture when

passaged repeatedly at a low cell density (Nugent & Iozzo, 2000).

CHAPTER III

METHODOLOGY

3.1 Construction of (GAGAGS)$_n$ -tagged GFP expression plasmid

Green fluorescent protein (GFP) amino acid sequence was translated into DNA sequence considering bacteria codon usage. For construction of pMALc5x-TEV-GFP expression plasmid, primer A and primer H (Table18 in appendix) were used in polymerase chain reaction (PCR) to amplify the GFP gene from pEGFP-N1. The PCR reaction recipe contained as follows: 0.5 ng/μL pEGFP-N1, 0.25 μM forward primer A and reverse primer H (primer sequences are shown in table18 in appendix), 0.2 mM dNTP, 5% v/v Dimethyl sulfoxide (DMSO), 0.02 U/μL Phusion hot-start polymerase, and the volume of UP water adjusted to an appropriate volume. The PCR reaction was introduced to Thermo Scientific Arktik Thermal Cycler, the PCR condition as follows: initial denaturing of 98 °C for 30 seconds; 25 cycles of 98 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds; and one final extension cycle of 72 °C 5 minutes. The PCR product was purified by agarose gel electrophoresis. The PCR

sample was mixed with 6x Gel Loading Dye Purple (Biolab) before adding (30 μL) into the well of 1% agarose gel in 1x TAE buffer with Hydra Green dye. The 1 kb Gold Bio DNA marker (5 μL) was added in the first lane. DNA separation condition was constant voltage at 100 V for 45 minutes. After the separation had finished, the gel was visualized under the UV light and cut gel between 750 and 1000 band size and cleaned up using Bio Fact Hi geneTM gel & PCR Purification. After purifying the PCR product, The PCR product, and pMALc5x construct plasmid was cut with 5 U/μL NcoI and 10 U/μL BamHI restriction enzyme and incubated at 37 °C for 1 hour. Then, the constructed plasmid was dephosphorylated with 1 U/μL shrimp alkaline phosphatase (rSAP) and incubated at 37 °C for 30 minutes. The digested PCR product and pMALc5x were cleaned up with Bio Fact Hi geneTM gel & PCR Purification. The purify digested PCR product and pMALc5x were cloned using a molar ratio of 1:3 construct vector to PCR product by 80 U/μL T4 DNA ligase and incubated at 4 °C for 4-6 hours. The ligation solution was transformed into *E. coli* DH10β using heat shock method. Ligation solution (5 μL) was added into the 50 μL of competent *E.*

coli DH10β, and incubated for 30 minutes on ice. The mixture was dipped into 42 °C water for 30 seconds, then the tube was set on ice for 2 minutes. The transformed cell was added to the 250 μL of SOC, and shook at 37 °C, 250 rpm for 1 hour. Lastly, 300 μL of the mixture was spread on LB agar plates that contain 100 μg/ml ampicillin and incubated at 37 °C for 16 – 18 hours, and colonies occurred.

The single colony was picked into a tube of LB broth (5 ml) containing the same concentration of antibiotics and shook at 37 °C, 250 rpm overnight. PCR was then performed to confirm the successful construction *tev-gfp* gene into pMALc5x plasmid. For each reaction, cell culture (1 μL) was added into 9 μL of UP water, boiled the sample on 98 °C for 3 minutes. The boiled sample was used as the template for the PCR. The PCR reaction composition was as follows: 2 mM MgCl2, 0.2 mM dNTP, 0.5 μM forward primer 1 and reverse primer 1, 0.01 U/μL Taq DNA polymerase, and 10 μL boiled sample. The PCR products were analyzed by agarose gel electrophoresis. The sample was mixed with 6x Gel Loading Dye Purple (Biolab) before adding (20 μL) into the well of 1% agarose gel in 1x TAE buffer with Hydra

Green dye. The ladder 1 kb Gold Bio DNA marker (5 μL) was added in the first well.

DNA separation condition was constant voltage at 100 V for 45 minutes. After the

separation had finished, the gel was visualized under UV light. The positive clones

would appear between 750 bases to 1000 bases band size. The positive colony cell

culture (1.5 ml) was extracted of plasmid using the method and materials suggested

in the BioFactTM Plasmid Mini Prep Kit. Finally, the positive plasmid concentration

was determined by spectrophotometry at A_{260} before sequencing to confirm the

correct construction plasmid.

Other expression plasmids were generated a similar manner. Briefly, for the

pMALc5x-TEV-GAGAGS-GFP expression plasmid, primers B and H were used to amplify

the pMALc5x-TEV-GFP template. For the pMALc5x-TEV-(GAGAGS)$_2$-GFP expression

plasmid, primers C and H were used to amplify the pMALc5x-TEV-GAGAGS-GFP

template. For the pMALc5x-TEV-(GAGAGS)$_3$-GFP expression plasmid, primers D and H

were used to amplify the pEGFP-N1 template. For pMALc5x-TEV-(GAGAGS)$_4$-GFP

expression plasmid, primers E and H were used to amplify the pMALc5x-TEV-

(GAGAGS)$_3$-GFP template to obtain the TEV-(GAGAGS)$_4$-GFP PCR product. For

pMALc5x-TEV-(GAGAGS)$_5$-GFP, the TEV-(GAGAGS)$_4$-GFP PCR product was re-amplified

with primers F and H to obtain TEV-(GAGAGS)$_5$-GFP for cloning. The TEV-(GAGAGS)$_4$-

GFP PCR product was also re-amplified with primers G and H to obtain the TEV-

(GAGAGS)$_6$-GFP PCR product for cloning. The nucleotide sequences of all expression

plasmids were confirmed by DNA sequencing.

3.2 (GAGAGS)$_n$ tagged GFP protein expression and purification

The suspension of competent *E. coli* tuner (DE3) (50 μL) was incubated on

ice and 3 μL of pMALc5x-TEV-(GAGAGS)$_n$-GFP, 20 μL of KCM, and 27 μL of sterile

distilled water were added. After incubation on ice for 30 minutes, the mixture was

heat-shocked at 42 ^{o}C for 30 seconds, then on ice for 2 minutes. SOC media (250 μL)

was added to the mixture before shook at 37 ^{o}C for 1 hour. The cells (100 μL) were

spread on an LB agar plate containing 100 μg/ml of ampicillin. This plate was

incubated at 37 oC for 16-18 hours. *E. coli* tuner (DE3) that was transformed with

pMALc5x-TEV-(GAGAGS)$_n$-GFP has grown in 100 ml Terrific Broth with 100 μg/ml

ampicillin at 37 oC for 16 – 18 hours. Cell cultures were grown in 500 ml Terrific

Broth using 3% inoculum of starter at 37 oC until OD_{600} occurred around 0.4 – 0.6.

IPTG was added to cell culture at 1 mM final concentration. The culture was

incubated at 30 °C for 6 hours. Finally, the cell culture was harvested by

centrifugation at 6,000 xg for 10 minutes and frozen the cell pellet at -80 °C.

The cell pellet was resuspended in 30 ml of buffer A (25 mM HEPES, 150 mM

NaCl, and 25 mM Imidazole; pH 7.5). Lysozyme (10 mg/ml, 100 μL) was added, and

the mixture was incubated at room temperature for 30 minutes, then PMSF (500 μL)

was added. Then, the mixture was sonicated using BANDELIN SONOPULS HD 2200

homogenizer, KE76 probe, for 15 seconds, 30% of pulse cycle, and 40% of power

and disrupted the mixture until the mixture became less viscous. The suspension

was centrifuged at 40,000 xg, 4 °C for 30 minutes. The supernatant was loaded onto

3-ml Ni-NTA agarose Beads column equilibrated in buffer A. Non-specific binding

protein was washed with buffer A. Elution was performed single step with buffer A

changing 25 mM to 250 mM Imidazole. MBP-TEV-(GAGAGS)$_n$-GFP was digested with

TEV proteinase and 100 mM DTT at 4 °C for 24 hours or until digested completely

detected by SDS-gel electrophoresis. After that, the digestive protein was dialyzed in

buffer A and purify again in Ni-NTA Agarose Beads column. The purify protein was

dialyzed against 20 mM Tris, pH 7.5. The (GAGAGS)$_n$-GFP was loaded onto 1 ml Q-

Sepharose column (GE Healthcare) pre-equilibrated with buffer B (20 mM Tris, pH 7.0)

and washed with buffer B (20 mM Tris 60 mM NaCl, pH 7.0) for removal TEV

proteinase. The purify (GAGAGS)$_n$-GFP peptide was eluted with buffer B (20 mM Tris

100 mM NaCl). The purity of (GAGAGS)$_n$-GFP was checked by using SDS-PAGE and

Coomassie Brilliant blue straining. The pure (GAGAGS)$_n$-GFP peptide was dialyzed

against 20 mM Tris, pH 7.0 overnight. The dialyzed protein was concentrated using

centrifugal filter before determining concentration by Bradford assay.

3.3 Preparation of silk fibroin solution

Silk cocoon "Nangnoi Srisaket" of thai silkworm *B. mori* was cleaned to

remove the outer layer of irregular fibers on the cocoons such as dirt, dead silkworm,

and other visible contaminants. The clean cocoon was dissolved in 0.2 Na_2CO_3

solution for remove sericin protein. The solution was heated until its boiling point

and then cleaned silk cocoon (40 g) was added into boiling solution. The sericin was

removed after 20 minutes. The silk fibers were washed with distill water until the

water appear colorless and dried at room temperature for 2 days. After that, the

dried silk fibers were sterilized with steam and pressure for 15 minutes. The sterilized

silk fibers (4 g) were dissolved in 9.3 M LiBr solution and placed in 60 °C oven. The

amber viscous of dissolved silk fibers was occurred after 4 hours. To remove the LiBr

from SF solution, the solution was dialyzed in distil water for 48 hours using dialysis

bag with 12-16 MWCO. The dialysis solution was centrifuged for remove the

impurities at 9,000 rpm, 4 °C for 20 minutes. Finally, the SF solution was measured

concentration in percentage of weight by volume.

3.4 Silk fibroin gelation time detection

The SF stock solution was prepared at 4% (w/v) concentration by dilution

with deionized water, and the $(GAGAGS)_n$-GFP stock solutions were prepared to 100

μg/ml concentration. The SF stock solution (100 μl) and the $(GAGAGS)_n$-GFP stock

solution (100 μl) were mixed in a well of a 96-well plate. The solution was

incubated at 37°C until the gelation occurred. The gelation kinetic was determined

by tracking the absorbance of 550 nm light because SF absorbs light best at 550 nm

when gelled but barely absorbs 550 nm before gelled. Microplate reader (absorbance mode) was used to determine the sol-gel transition.

3.5 *In vitro* GFP release

To determine the release profile of GFP as a model drug, the SF hydrogels were collected from 96-well plate. The amount of GFP release from SF hydrogel was measured by its absorbance at 395 nm using spectrophotometer. The hydrogel was cut into cubic shape (2*2*2 mm) and weighed around 20 mg of each sample. The SF hydrogel was submerged in 1 ml of deionized water in cuvette. The absorbance values at 395 nm were recorded every 10 minutes within the first 2 hours, and then every 12 hours until the absorbance values no longer changed.

3.6 *In vitro* enzymatic degradation of silk fibroin hydrogel

The cubic sample of SF hydrogel was cut in 4 mm*4 mm* 4 mm and weighted around 40 mg. The sample cubic were submerged in PBS buffer (10 ml) that contained proteinase XIV (1 U/ml) and incubated at 37 °C for 30 days. The buffer solution was taken out and replaced daily by fresh buffer with enzyme. At a specific

time point, the buffer was removed. The retrieved hydrogel cube was rinsed with

distilled water, and the weight of hydrogel was determined. The weight ratio was

calculated as follows equation 1.

$$\% \ of \ gel \ weight \ remaining = \frac{Wt}{w0}x100 \qquad \text{(Equation 1)}$$

W_0 = Weight of SF hydrogel before added enzyme

W_t = Weight of SF hydrogel remaining at every 2 days interval

3.7 SF hydrogel structure characterization

3.7.1 Lyophilized silk fibroin assemble with $(GAGAGS)_n$-GFP-H_6 peptide

hydrogel

Lyophilized-gel was prepared by lyophilized in a freeze dryer. The hydrogel

sample was first prefreezed in liquid nitrogen for quick-frozen to avoid structural

changes, then was stored at -20 °C overnight before lyophilization. The drying SF

hydrogel sample was performed at -54 °C, 0.1 mbar (vacuum) for 72 hours. Finally,

lyophilized-gel samples were stored at 4 °C before characterization.

3.7.2 X-ray diffraction (XRD) of SF lyophilized gel

The XRD analysis of the hydrogels was performed using X-ray diffractometer

(Rigaku, Smartlab 30 kV) equipped with a fixed monochromator and CuKα radiation

source. All patterns were measured at 2θ in the range of 5-40°. The diffraction at

the 2θ angle of 19.5 – 22.5° is represented of the SF crystalline fraction.

3.7.3 Fourier-transform infrared (FTIR) spectroscopy

The lyophilized-gel samples were first placed onto IR-transparent discs and

then analyzed using sn FTIR microscopic spectrometer (Nicolet iS50 FTIR, Thermo

Fisher Scientific, Waltham, MA, USA). The spectrum of infrared was collected

between 4000 to 400 cm^{-1} in a transmission mode. The absorption of amide I is 1650

cm^{-1} for the random coils and 1630 cm^{-1} for β-sheets. The absorption of amide II is

1540 cm^{-1} for the random coils and 1520 cm^{-1} for the β-sheets.

3.8 Construction of (GAGAGS)$_n$-tagged bFGF expression plasmid

For the construction of the pET28b-bFGF expression plasmid for produced

the native bFGF that didn't have tag or any linker, primers I and K (table 18 in

appendix) were used in polymerase chain reaction (PCR) to amplify the GFP gene

from cDNA of Hela cells and cloned into the NcoI and HindIII sites of pET28b. The

PCR reaction recipe contained as follows: 0.5 ng/μL GFP cDNA, 0.25 μM forward

primer1 and reverse primer1 (primer sequences are shown in the appendix), 0.2 mM

dNTP, 5% v/v Dimethyl sulfoxide (DMSO), 0.02 U/μL Phusion hot-start polymerase,

and the volume of UP water adjusted to an appropriate volume. The PCR reaction,

ligation, transformation, and collected positive clones were followed as describes in

methodology 3.1. For the pMALc5x-H_6-TEV-bFGF expression plasmid was generated in

a similar manner, primers J and K were used to amplify the cDNA of Hela cells

templated. And the pMALc5x-H_6-TEV-(GAGAGS)$_6$-bFGF expression plasmid was

purchased from Biomatik.

3.9 bFGF and (GAGAGS)$_6$-bFGF expression and purification

3.9.1 IPTG optimization for bFGF and (GAGAGS)$_6$-bFGF expression

The transformed *E. coli* tuner (DE3) with pET28b-bFGF was cultured in LB

(containing 50 μg/ml of kanamycin) until the OD_{600} reached 0.6. And the transformed

cells with pMALc5x-H$_6$-TEV-bFGF, and pMALc5x-H$_6$-TEV-(GAGAGS)$_6$-bFGF were cultured

in LB (containing 100 μg/ml of ampicillin) until the OD$_{600}$ reached 0.6 too. The

cultures (2 ml) were aliquoted and IPTG were added (0 – 1 mM) to induce protein

expression for 4 hours. The cell pellets were collected before adding 500 μL of the

detergent solution (20 mM Tris, pH 7.0, 0.5% octylthioglucoside, 5 μg of the 10

mg/ml lysozyme, and 0.25 μL of 5 U/μL benzonase) to break the cells for 15

minutes. After centrifugation, the supernatants were collected into new tube while

water 500 μL were added to the remaining pellet. Loading buffer with DTT (6x) were

added to the supernatant and cell debris before boiling. The protein samples were

analyzed by SDS-PAGE electrophoresis.

3.9.2 bFGF and (GAGAGS)$_6$-bFGF expression using *Escherichia coli* Tuner

(DE3)

The transformed cells containing pET28b-bFGF was picked into 50 ml of of

kanamycin-containing terrific broth and the transformed cells with pMALc5x-H$_6$-TEV-

bFGF, and pMALc5x-H$_6$-TEV-(GAGAGS)$_6$-bFGF were picked into 50 ml ampicillin terrific

broth as the starter culture for large scale production then incubated at 37 °C for 16

– 18 hours. For bFGF expressed from pET28b-bFGF, four flasks of 500 ml of terrific

broth containing 50 μg/ml kanamycin were inoculated with 15 ml of the starter

culture. And bFGF and (GAGAGS)$_6$-bFGF from pMALc5x plasmid production, four flasks

of 500 ml ampicillin-terrific broth were inoculated with 15 ml of the culture starter.

All flasks were shaken at 37 °C until OD$_{600}$ reached to 0.4-0.6. The IPTG was added

following the optimized concentration. Protein expressions were carried out at 30 °C

for 6 hours. Finally, the cell pellets were obtained by centrifugation at 6,000 xg for 10

minutes. The pellets were stored at -80 °C.

3.9.3 Purification of bFGF peptide expressed from pET28b-bFGF plasmid

The cell pellet was resuspended in 30 ml in 20 mM Tris, 150 mM NaCl pH 7.5.

Lysozyme (10 mg/ml, 100 μL) was added, and the mixture was incubated at room

temperature for 30 minutes, then PMSF (500 μL) was added. Then, the mixture was

sonicated using BANDELIN SONOPULS HD 2200 homogenizer, KE76 probe, for 15

seconds, 30% of pulse cycle, and 40% of power and disrupted the mixture until the

mixture became less viscous. The suspension was centrifuged at 40,000 xg, 4 °C for

30 minutes. The supernatant (28.5 ml) mixed with 0.25% of 5% PEI (1.5 ml) and

incubated the solution on ice for 5 minutes. The solution was centrifuged at 6,000 xg

for 10 minutes and kept the supernatant. After that, the supernatant was dialyzed in

20 mM Tris, pH 7.5. A 2 M of ammonium sulfate $((NH_4)_2SO4))$ was added to the

solution and kept at room temperature for 5 minutes. After that, the solution was

centrifuged at 40,000 xg 4 °C for 15 minutes and kept the supernatant solution. The

supernatant was loaded onto Phenyl Hydrophobic column equilibrated in 20 mM

Tris, 2 M $(NH_4)_2SO_4$, pH 7.5. Non-specific binding protein was washed with same

buffer. Elution was performed gradient step with 20 mM Tris, changing 2 M to 1 M,

0.5 M, and 0 M $(NH_4)_2SO_4$. The elution protein fractions were checked by SDS-PAGE

and Coomassie Brilliant blue straining. The elution fraction that had target protein

were pooled and dialyzed against 20 mM Tris, pH 7.5. Finally, the dialysis solution

was purified by fast protein liquid chromatography (FPLC) with SP column that

equilibrated in 20 mM Tris, pH 7.5. Elution was performed gradient step with 60% of

20 mM Tris, 1 M NaCl, pH 7.5. The purified protein fractions were checked by SDS-

PAGE and Coomassie Brilliant blue straining. The pure fractions were pooled and dialyzed against 20 mM Tris, pH 7.5. The dialyzed protein was concentrated using centrifugal filter and determined concentration using A_{280} following by Beer's law

$$A = \varepsilon bc$$

Where A is absorbance.

ε is extinction coefficient or molar absorptivity, 15,930 M^{-1} cm^{-1} for bFGF as estimated from protein sequence by ExPASy

b is the path length, 1 cm.

c is the concentration of protein in solution.

3.9.4. Purification of bFGF and (GAGAGS)$_6$-bFGF expressed from pMALc5x plasmid

The cell pellet that have bFGF or (GAGAGS)$_6$-bFGF was resuspended in 30 ml of buffer A (25 mM HEPES, 150 mM NaCl, and 25 mM Imidazole; pH 7.5). Lysozyme (10 mg/ml, 100 μL) was added, and the mixture was incubated at room temperature for 30 minutes, then PMSF (500 μL) was added. Then, the mixture was sonicated

using BANDELIN SONOPULS HD 2200 homogenizer, KE76 probe, for 15 seconds, 30% of pulse cycle, and 40% of power and disrupted the mixture until the mixture became less viscous. The suspension was centrifuged at 40,000 xg, 4 °C for 30 minutes. The supernatant was loaded onto 3-ml Ni-NTA agarose Beads column equilibrated in buffer A. Non-specific binding protein was washed with buffer A. Elution was performed single step with buffer A changing 25 mM to 250 mM Imidazole. MBP-H$_6$-TEV-bFGF or MBP-H$_6$-TEV-(GAGAGS)$_6$-bFGF was digested with TEV proteinase and 100 mM DTT at 4 °C for 24 hours or until digested completely detected by SDS-gel electrophoresis. After that, the digestive protein was dialyzed in buffer A and purify again in Ni-NTA Agarose Beads column and kept the wash fraction. The purify protein was dialyzed against 20 mM Tris, pH 7.5. The dialysis solution was purified by fast protein liquid chromatography (FPLC) with SP column that equilibrated in 20 mM Tris, pH 7.5. Elution was performed gradient step with 60% of 20 mM Tris, 1 M NaCl, pH 7.5. The purified protein fractions were checked by SDS-PAGE and Coomassie Brilliant blue straining. The pure fractions were pooled and dialyzed against 20 mM Tris, pH 7.5. The dialyzed protein was concentrated using

centrifugal filter and determined concentration using A_{280} following by Beer's law as described in methodology 3.9.3.

3.10 Cell culture

NIH3T3 fibroblast cells were maintained in Dulbecco's modified engle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C. The subculture was performed when confluency reached approximately 80-90% using trypsin-EDTA solution to detach the adherent cells.

3.11 Optimized concentration of bFGF and (GAGAGS)$_6$-bFGF on proliferation of NIH-3T3

The fibroblast cells were seeded at 1×10^3 cells/ml in 96-well plates and incubated in a humidified 5% CO_2 atmosphere at 37 °C overnight for cell attachment. The DMEM medium was change and treated with various concentration (0, 1, 10, 100 ng/ml) of bFGF and (GAGAGS)$_6$-bFGF and incubated for 24 hours. After that, the media was discarded and added the new medium containing MTT solution (10

mg/ml in sterilized water) at the same ratio. The cells were incubated until purple

crystal was occurred. Finally, the purple crystals were dissolved by isopropanol and

the resulting-colored solution is quantified by measuring of its absorbance at 570 nm

light using multi-wells spectrophotometer.

3.12. SF hydrogel assembly with optimal concentration of bFGF and (GAGAGS)$_6$-bFGF formation

The SF solution was diluted to 4% w/v concentration and sterilized with

steam and pressure for 15 minutes and vortexing. After that, the sterilized SF solution

(2 ml) mixed with the optimal concentration of bFGF and (GAGAGS)$_6$-bFGF in 6 wells

plate. The solution was incubated at 37 °C until the hydrogel was occurred.

3.13 *In vitro* bFGF release

The fibroblast cells were seeded at 1×10^3 cells/ml in 6-well plates and

incubated in DMEM media at 37 °C overnight for cell attachment. The DMEM

medium was change and putted four SF containing optimal concentration of growth

factor hydrogel cubic (2 mm*2 mm*2 mm) and placed at the corner of well and

incubated for 16 days. Medium was changed every day. Every 2 days interval, the

MTT assay was conducted as described in methodology 3.11.

CHARTER IV

RESULTS

4.1. Construction of (GAGAGS)$_n$ -tagged GFP expression plasmids

The DNA sequences coding for the SF repeating unit (GAGAGS) ranging from zero to six repeat unit ((GAGAGS)$_n$, n = 0-6) were fused to the DNA sequence of the green fluorescent protein (GFP). TEV-(GAGAGS)$_n$-GFP fragments was amplify by polymerase chain reaction (PCR). Detection of the PCR product was analyzed by agarose gel electrophoresis (Fig 5) that TEV-(GAGAGS)$_n$-GFP PCR product showed band size between 800 and 900 bps. After purified PCR product, these PCR products and pMALC5x were cut with *NcoI* and *BamHI* restriction enzyme and cloned by ligation process. Then, the ligation reaction was transformed to *E. coli* DH10β. To confirm the cloning result, the transformed *E. coli* DH10β were checked that the TEV-(GAGAGS)$_n$-GFP fragments were constructed into the expression plasmid by colony PCR. The positive construction plasmids were extracted and confirmed by nucleic acid sequencing.

The nucleotide sequences analysis showed eight construct plasmid ranging from zero to seven repeat unit (pMALc5x-TEV-(GAGAGS)$_n$-GFP, n = 0-7) pMALc5x-TEV-(GAGAGS)$_7$-GFP was isolated as a byproduct during the construction of pMALc5x-TEV-(GAGAGS)$_6$-GFP, likely by mis-priming during PCR process. The nucleic acid sequence of all gene constructed in pMALc5x plasmid was shown in table 7
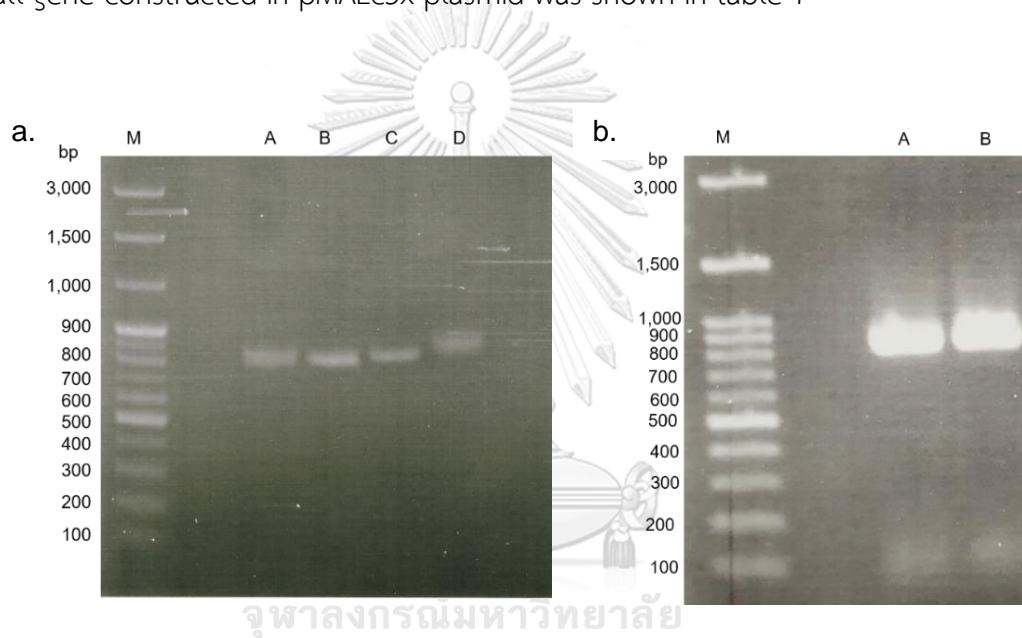
Figure 5: TEV-(GAGAGS)$_n$-GFP PCR product in 1% agarose gel. a). M = DNA marker, A = TEV-GFP fragment, B = TEV-GAGAGS-GFP fragment, C = TEV-(GAGAGS)$_3$-GFP fragment, D = TEV-(GAGAGS)$_6$-GFP fragment. b). M = DNA marker, A = TEV-(GAGAGS)$_2$-GFP fragment, B = TEV-(GAGAGS)$_5$-GFP fragment.

Table 7: Nucleic acid sequence of TEV-(GAGAGS)$_n$-GFP fragment after sequencing analysis

gene	Nucleic acid sequence								
TEV-GFP	catatgtcca	tgggcgaaaa	cctgtatttt	caggttcat	gagcaagggc	gaggagctgt	tcaccggggt	ggtgcccatc	
	ctggtcgagc	tggacggcga	cgtaaacggc	cacaagttca	gcgtgtccgg	cgagggcgag	ggcgatgcca	cctacggcaa	gctgaccctg
	aagttcatct	gcaccaccgg	caagctgccc	gtgccctggc	ccaccctcgt	gaccaccctg	acctacggcg	tgcagtgctt	cagccgctac
	cccgaccaca	tgaagcagca	cgacttcttc	aagtccgcca	tgcccgaagg	ctacgtccag	gagcgcacca	tcttcttcaa	ggacgacggc
	aactacaaga	cccgcgccga	ggtgaagttc	gagggcgaca	ccctggtgaa	ccggcatcgag	ctgaagggca	tcgacttcaa	ggaggacggc
	aacatcctgg	ggcacaagct	ggagtacaac	tacaacagcc	acaacgtcta	tatcatggcc	gacaagcaga	agaacggcat	caaggtgaac
	ttcaagatcc	gccacaacat	cgaggacggc	agcgtgcagc	tcgcccacca	ctaccagcag	aacaccccca	tcggcgacgg	ccccgtgctg
	ctgcccgaca	accactacct	gagcacccag	tccgccctga	gcaaagaccc	caacgagaag	cgcgatcaca	tggtcctgct	ggagttcgtg
	accgccgccg	ggatcactct	cggcatggac	gagctgtaca	aggcggccgc	actcgagcac	caccaccacc	accactaata	a
TEV-GAGAGS-GFP	catatgtcca	tgggcgaaaa	cctgtatttt	cagggtggtg	ctggtgctgg	ctctggtgct	ggtgctggct	catcaggtat	ggtggagcaag
	ggcgaggagc	tgttcaccgg	ggtggtgccc	atcctggtcg	agctggacgg	cgacgtagac	ggccacaagt	tcagcgtgtc	cggcgagggc
	gagggcgatg	ccacctacgg	caagctgacc	ctgaagttca	tctgcaccac	cggcaagctg	cccgtgccct	ggcccacccc	cgtgaccacc
	ctgacctacg	gcgtgcagtg	cttcagccgc	tacccccgac	cacatgaagc	agcacgactt	cttcaagtcc	gccatgcccg	aggctacgtc
	caggagcgca	ccatcttctt	caaggacgac	ggcaactaca	agacccgcgc	cgaggtgaag	ttcgagggcg	acaccctggt	gaaccgccatc
	gagctgaagg	gcatcgactt	caaggaggac	ggcaacatcc	tggggcacaa	gctggagtac	aactacaaca	gccacaacgt	ctatatcatg
	gccgacaagc	agaagaacgg	catcaaggtg	aacttcaaga	tccgccacaa	cgacgagtac	tgggagcaca	gccacaacgt	gaaccgccatc
	gccgacaagc	agaagaacgg	catcgaggac	tccgccacaa	aacttcaaga	catcgaggac	ggcagcgtgc	agctcgccga	ccactaccag

cagaacaccc	ccatcggcga	cggccccgtg	ctgctgcccg	acaaccacta	cctgagcacc	cagtccgccc	tgagcaaaga	
ccccaacgag	aagcccgatc	acatggtcct	gctggagttc	gtgaccgccg	ccgggatcac	tctcggcatg	gacgagctgt	acaaggcggc
cgcactcgag	caccaccacc	accaccactg	ataa					

TEV-(GAGAGS)₂-GFP

catatgtcca	tgggcgaaaa	cctgtatttt	caggggtggtg	ctggtgctgg	ctcaggtgct	ggtgctggct	caggtgccgg	atctggttca
tcaggtatgg	tgagcaaggg	cgaggagctg	ttcaccgggg	tggtgcccat	cctggtcgag	ctggacggcg	acgtaaacgg	ccacaagttc
agcgtgtccg	gcgagggcga	gggcgatgcc	acctacggca	agctgaccct	gaagttcatc	tgcaccaccg	gcaagctgcc	cgtgccctgg
cccaccctcg	tgaccaccct	gacctacggc	gtgcagtgct	tcagccgcta	ccccgaccac	atgaagcagc	acgacttctt	caagtccgcc
atgcccgaag	gctacgtcca	ggagcgcacc	atcttcttca	aggacgacgg	caactacaag	acccgcgccg	aggtgaagtt	cgagggcgac
accctggtga	accgcatcga	gctgaagggc	atcgacttca	aggaggacgg	caacatcctg	gggcacaagc	tggagtacaa	ctacaacagc
cacaacgtct	atatcatggc	gacaagcag	aagaacggca	tcaaggtgaa	cttcaagatc	cgccacaaca	tcgaggacgg	cagcgtgcag
ctcgccgacc	actaccagca	gaacaccacc	atcggcgacg	gccccgtgct	gctgcccgac	aaccactacc	tgagcaccca	gtccgccctg
agcatagacc	ccaacgagaa	gcgcgatcac	atggtcctgc	tggagttcgt	gaccgccgcc	gggatcactc	tcggcatgga	cgagctgtac
aaggcggccg	cactcgagca	ccaccaccac	caccactgat	aa				

TEV-(GAGAGS)₃-GFP

catatgtcca	tgggcgaaaa	cctgtatttt	caggggtggtg	ctggtgctgg	ctcaggtgct	ggtgctggat	ctggggcagg	agcaggaagt
ggtgctggct	ctggttcatc	aggtatggtg	agcaagggcg	aggagctgtt	caccggggtg	gtgcccatcc	tggtcgagct	ggacggcgac
gtaaacggcc	acaagttcag	cgtgtccggc	gagggcgagg	gcgatgccac	ctacggccaag	ctgaccctga	agttcatctg	caccaccggc
aagctgcccg	tgccctggcc	caccctcgtg	accacccctga	cctacggcgt	gcagtgcttc	agccgctacc	ccgaccacat	gaagcagcac
gacttcttca	agtccgccat	gcccgaaggc	tacgtccagg	agcgcaccat	cttcttcaag	gacgacggca	actacaagac	ccgcgccgag
gtgaagttcg	agggcgacac	cctggtgaac	cgcatcgagc	tgaagggcat	cgacttcaag	gaggacggca	acatcctggg	gcacaagctg

	gagtacaact acaacagcca caacgtctat atcatggccg acaagcagaa gaacggcatc aaggtgaact tcaagatccg
	ccacaacatc gaggacggca gcgtgcagct cgccgaccac taccagcaga acacccccat cggcgacggc cccgtgctgc tgcccgacaa
	ccactacctg agcacccagt ccgccctgag caaagacccc aacgagaagc gcgatcacat ggtcctgctg gagttcgtga ccgccgccgg
	gatcactctc ggcatggacg agctgtacaa ggcggccgca ctcgagcacc accaccacca ccactgataa
TEV-(GAGAGS)₄-GFP	catatgtcca tgggcgaaaa cctgtatttt cagggtggag caggagct atcaggagct ggagctggat caggagctgg
	agctggaagt ggtgctggtg ctggttcagg tgcaggctcc ggatcatcag gtatggtgag caagggcgag gagctgttca
	ccggggtggt gcccatcctg gtcgagctgg acggcgacgt aaacggccac aagttcagcg tgtccggcga gggcgagggc
	gatgccacct acggcaagct gaccctgaag ttcatctgca ccaccggcaa gttgcccgtg ccctggccca ccctcgtgac
	caccctgacc tacggcgtgc agtgcttcag ccgctacccc gaccacatga agcagcacga cttcttcaag tccgccatgc
	ccgaaggcta cgtccaggag cgccaccatct tctttcaagg gaccacatga tacaagaccc gcgccgaggt gaagttcgag
	ggcgacaccc tggtgaaccg catcgagctg aagggcatcg acttcaagga ggacggcaac atcctggggc acaagctgga
	gtacaactac aacagccaca acgtctatat catggccgac aagcagaaga acggcatcaa ggtgaacttc aagatccgcc
	acaacatcga ggacggcagc gtgcagctcg ccgaccacta ccagcagaac acccccatcg gcgacggccc cgtgctgctg
	cccgacaacc actacctgag cacccagtcc gccctgagca aagaccccaa cgagaagcgc gatcacatgg tcctgctgga
	gttcgtgacc gccgccggga tcactctcgg catggacgag ctgtacaagg cggccgcact cgagcaccac caccaccacc
	actgataa			
TEV-(GAGAGS)₅-GFP	catatgtcca tgggcgaaaa cctgtatttt cagggtggag agcaggagca ttctggggcc ggcgctggct cgggtgctgg
	tgctggctca ggtgctggtg ctggatctgg ggaagtggtg ggatctggct ctggctctgg ttcatcaggt atggtgagca
	agggcgagga gctgttcacc gggtggttgc ccatcctggt cgagctggac ggcgacgtaa acggccacaa gttcagcgtg

tccggccgagg	gcgaggggcga	tgccacctac	ggcaagctga	ccctgaagtt	catctgcacc	accggcaagc	tgcccgtgcc
ctggcccacc	ctcgtgacca	ccctgaccta	cggcgtgcag	tgcttcagcc	gctaccccga	ccacatgaag	cagcacgact
tcttcaagtc	cgccatgccc	gaaggctacg	tccaggagcg	caccatcttc	ttcaaggacg	acggcaacta	caagacccgc
gccgaggtga	agttcgaggg	cgacaccctg	gtgaaccgca	tcgagctgaa	gggcatcgac	ttcaaggagg	acggcaacat
cctggggcac	aagctggagt	acaactacaa	cagccacaac	gtctatatca	tggccgacaa	gcagaagaac	ggcatcaagg
tgaacttcaa	gatccgccac	aacatcgagg	acggcagcgt	gcagctcgcc	gaccactacc	agcagaacac	ccccatcggc
gacggccccg	tgctgctgcc	cgacaaccac	tacctgagca	cccagtccgc	cctgagcaaa	gaccccaacg	agaagcgcga
tcacatggtc	ctgctggagt	tcgtgaccgc	cgccgggatc	actctcggca	tggacgagct	gtacaaggcg	gccgcactcg
agcaccacca	ccaccaccac	tgataa					

	catatgtcca	tgggcgaaaa	cctgtatttt	caggggtggtg	ctggtgctgg	ctctggagca	ggagcaggat	ctggggccgg
	cgctggctcg	ggtgctggtg	ctggctcagg	tgctggtgct	ggatctggag	caggagcagg	aagtggtgct	ggctctggtt
	catcaggtat	ggtgagcaag	ggcgaggagc	tgtttcaccgg	ggtggttgccc	atcctggtcg	agctggacgg	cgacgtaaac
	ggccacaagt	tcagcgtgtc	cggcgaggggc	gagggcgatg	ccacctacgg	caagctgacc	ctgaagttca	tctgcaccac
TEV-	cggcaagttg	cccgtgccct	ggcccaccct	cgtgaccacc	ctgacctacg	gcgtgcagtg	cttcagccgc	taccccgacc
(GAGAGS)6-GFP	acatgaagca	gcacgacttc	ttcaagtccg	ccatgcccga	aggctacgtc	caggagcgca	ccatcttctt	caaggacgac
	ggcaactaca	agacccgcgc	cgaggtgaag	ttcgaggggcg	acaccctggt	gaaccgcatc	gagctgaagg	gcatcgactt
	caaggaggac	ggcaacatcc	tggggcacaa	gctggagtac	aactacaaca	gccacaacgt	ctatatcatg	gccgacaagc
	agaagaacgg	catcaaggtg	aacttcaaga	catcgaggac	ggcagcgtgc	agctcgccga	ccactaccag	ccactaccag
	cagaacaccc	ccatcggcga	cggcccctg	acaaccacta	cctgagcacc	cagtccgccc	tgagcaaaga	tgagcaaaga

Name								
	ccccaacgag	aagcgcgatc	acatggtcct	gctggagttc	gtgaccgccg	ccgggatcac	tctcggcatg	gacgagctgt
	acaaggcggc	cgcactcgag	caccaccacc	accaccactg	ataa			
TEV-(GAGAGS)$_7$-GFP	catatgtcca	tgggcgaaaa	cctgtatttt	cagggtggag	caggagctgg	atcaggagct	ggagctggat	caggagctgg
	agctggaagt	ggtgctggtg	ctggctcagg	tgctggttct	ggatctggag	caggagcagg	aagtggtgca	ggagctggtt
	caggtgctgg	ctctggttca	tcaggtatgg	tgagcaaggg	cgaggagctg	ttcaccgggg	tggtgcccat	cctggtcgag
	ctggacggcg	acgtaaacgg	ccacaagttc	agcgtgtccg	gcgagggcga	gggcgatgcc	acctacggca	agctgaccct
	gaagttcatc	tgcaccaccg	gcaagctgcc	cgtgccctgg	cccaccctcg	tgaccaccct	gacctacggc	gtgcagtgct
	tcagccgcta	ccccgaccac	atgaagcagc	acgacttctt	caagtccgcc	atgcccgaag	gctacgtcca	ggagcgcacc
	atcttcttca	aggacgacgg	caactacaag	acccgcgccg	aggtgaagtt	cgagggcgac	accctggtga	accgcatcga
	gctgaagggc	atcgacttca	aggaggacgg	caacatcctg	gggcacaagc	tggagtacaa	ctacaacagc	cacaacgtct
	atatcatggc	cgacaagcag	aagaacggca	tcaaggtgaa	cttcaagatc	cgccacaaca	tcgaggacgg	cagcgtgcag
	ctcgccgacc	actaccagca	gaacacccccc	atcggcgacg	gccccgtgct	gctgcccgac	aaccactacc	tgagcaccca
	gtccgccctg	agcaaagacc	ccaacgagaa	gcgcgatcac	atggtcctgc	tggagttcgt	gaccgccgcc	gggatcactc
	tcggcatgga	cgagctgtac	aaggcggccg	cactcgagca	ccaccaccac	caccactgat	aa	

Table 8: Amino acid sequence of recombinant peptides

Recombinant peptides	Amino acid sequence
TEV-GFP-H6	MSMGENLYFQ GSSGMVSKGE ELFTGVVPIL VELDGDVNGH KFSVSGEGEG DATYGKLTLK FICTTGKLPVP WPTLVTTLTY GVQCFSRYPD HKQHDFFKSA MPEGYVQERT IFFKDDGNYK TRAEVKFEGD TLVNRIELKG IDFKEDGNIL GHKLEYNYNS HNVYIMADKQ KNGIKVNFKI RHNIEDGSVQ LADHYQQNTP IGDGPVLLPD NHYLSTQSAL SKDPNEKRDH MVLLEFTAAG ITLGMDELYK AAALEHHHHH H
TEV-GAGAGS-GFP-H6	MSMGENLYFQ GGAGAGSGAG SGSSGMVSKG EELFTGVVP LVELDGDVDG HKFSVSGEGE GDATYGKLTL KFICTTGKLP VPWPTLVTTL TYGVQCFSRY PDHMKQHDFF KSAMPEGYVQ ERTIFFKDDG NYKTRAEVKF EGDTLVNRIE LKGIDFKEDG NILGHKLEYN YNSHNVYIMA DKQKNGIKVN FKIRHNIEDG SVQLADHYQQ NTPIGDGPVL LPDNHYLSTQ SALSKDPNEK RDHMVLLEFV TAAPGITLGM DELYKAAALE HHHHHH
TEV-(GAGAGS)2-GFP-H6	MSMGENLYFQ GGAGAGSGAG AGSGAGSGSS GMVSKGEELF TGVVPILVEL DGDVNGHKFS VSGEGEGDAT YGKLTLKFIC TTGKLPVPWP TLVTTLTYGV QCFSRYPDHM KQHDFFKSAM PEGYVQERTI FFKDDGNYKT RAEVKFEGDT DFKEDGNILG HKLEYNYNSH NVYIMADKQK NGIKVNFKIR HNIEDGSVQL ADHYQQNTTI GDGPVLLLDN HYLSTQSALS IDPNEKRDHM VLLEFVTAAG ITLGMDELYK AAALEHHHHH H
TEV-(GAGAGS)3-GFP-H6	MSMGENLYFQ GGAGAGSGAG AGSGAGAGSG AGSGSSGMVS KGEELFTGVV PILVELDGDV NGHKFSVSGE GEGDATYGKL TLKFICTTGK LPVPWPTLVT TLTYGVQCFS RYPDHMKQHD FFKSAMPEGY VQERTIFFKD

	DGNYKTRAEV	KFEGDTLVNR	IELKGIDFKE	DGNILGHKLE	YNYNSHNVYI	MADKQKNGIK	VNFKIRHNIE
	DGSVQLADHY	QQNTTIGDGP	VLLLDNHYLS	TQSALSIDPN	EKRDHMVLLE	FVTAAGITLG	MDELYKAAAL
	EHHHHHH						
TEV-(GAGAGS)4-GFP-H6	MSMGENLYFQ	GGAGAGSGAG	AGSGAGAGSG	AGAGSGAGSG	SSGMVSKGEE	LFTGVVPILV	ELDGDVNGHK
	FSVSGEGEGD	ATYGKLTLKF	ICTTGKLPVP	WPTLVTTLTY	GVQCFSRYPD	HMKQHDFFKS	AMPEGYVQER
	TIFFKDDGNY	KTRAEVKFEG	DTLVNRIELK	GIDFKEDGNI	LGHKLEYNYN	SHNVYIMADK	QKNGIKVNFK
	IRHNIEDGSV	QLADHYQQNT	TIGDGPVLLL	DNHYLSTQSA	LSIDPNEKRD	HMVLLEFVTA	AGITLGMDEL
	YKAAALEHHH	HHH					
TEV-(GAGAGS)5-GFP-H6	MSMGENLYFQ	GGAGAGSGAG	AGSGAGAGSG	AGAGSGAGAG	SGAGSGSSGM	VSKGEELFTG	VVPILVELDG
	DVNGHKFSVS	GEGEGDATYG	KLTLKFICTT	GKLPVPWPTL	VTTLTYGVQC	FSRYPDHMKQ	HDFFKSAMPE
	GYVQERTIFF	KDDGNYKTRA	EVKFEGDTLV	NRIELKGIDF	KEDGNILGHK	LEYNYNSHNV	YIMADKQKNG
	IKVNFKIRHN	IEDGSVQLAD	HYQQNTTIGD	GPVLLLDNHY	LSTQSALSID	PNEKRDHMVL	LEFVTAAGIT
	LGMDELYKAA	ALEHHHHHHH					
TEV-(GAGAGS)6-GFP-H6	MSMGENLYFQ	GGAGAGSGAG	AGSGAGAGSG	AGAGSGAGAG	SGAGAGSGAG	SGSSGMVSKG	EELFTGVVPI
	LVELDGDVNG	HKFSVSGEGE	GDATYGKLTL	KFICTTGKLP	VPWPTLVTTL	TYGVQCFSRY	PDHMKQHDFF
	KSAMPEGYVQ	ERTIFFKDDG	NYKTRAEVKF	EGDTLVNRIE	LKGIDFKEDG	NILGHKLEYN	YNSHNVYIMA
	DKQKNGIKVN	FKIRHNIEDG	SVQLADHYQQ	NTTIGDGPVL	LLDNHYLSTQ	SALSIDPNEK	RDHMVLLEFV
	TAAGITLGMD	ELYKAAALEH	HHHHHH				
TEV-(GAGAGS)7-	MSMGENLYFQ	GGAGAGSGAG	AGSGAGAGSG	AGAGSGAGAG	SGAGAGSGAG	AGSGAGSGSS	GMVSKGEELF

GFP-H6	TGVVPILVEL	DGDVNGHKFS	VSGEGEGDAT	YGKLTLKFIC	TTGKLPVPWP	TLVTTLTYGV	QCFSRYPDHM
	KQHDFFKSAM	PEGYVQERTI	FFKDDGNYKT	RAEVKFEGDT	LVNRIELKGI	DFKEDGNILG	HKLEYNYNSH
	NVYIMADKQK	NGIKVNFKIR	HNIEDGSVQL	ADHYQQNTTI	GDGPVLLLDN	HYLSTQSALS	IDPNEKRDHM
	VLLEFVTAAG	ITLGMDELYK	AAALEHHHHHH	H			

4.2 (GAGAGS)$_n$ tagged GFP protein expression and purification

After plasmid construction and DNA sequence verification, the (GAGAGS)$_n$-GFP

peptide expressed using the *E. coli* host for large scale production. After (GAGAGS)$_n$-

GFP peptide large scale production, the peptide needed to be purified and

concentrated. First, the peptide supernatant from sonication was purified using Ni-

NTA agarose column. Then, TEV protease was added to MBP-TEV-(GAGAGS)$_n$-GFP

fraction for digested at TEV site. After that, the digestive peptide was purified again

with Ni-NTA agarose column. The (GAGAGS)$_n$-GFP peptide was purified with Q-

Sepharose column because these peptides had negative change. After concentration

using centrifugal filter, the peptide purity was checked by the SDS-PAGE and showed

the (GAGAGS)$_n$-GFP band around 25-28 kDa (Fig 6) and concentration was determined

by Bradford assay. The (GAGAGS)$_n$-GFP peptide concentration was calculated using

linear regression formula of standard curve (shown in appendix) and concentration of

this peptide was shown in table 9.
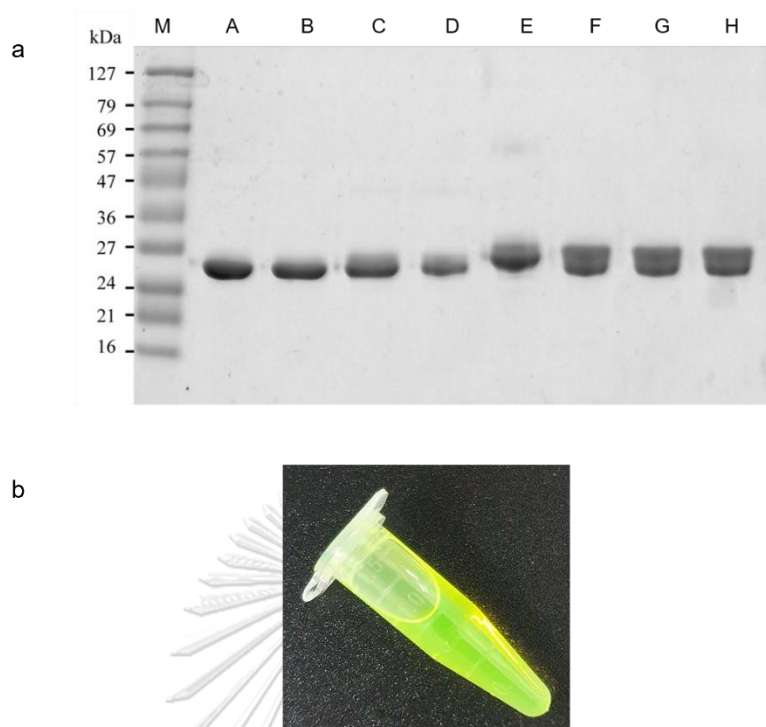
Figure 6: SDS-PAGE of purified recombinant (GAGAGS)$_n$-GFP peptide (a) and purified recombinant (GAGAGS)$_n$-GFP peptide (b). M = protein marker; A = GFP; B = GAGAGS-GFP; C = (GAGAGS)$_2$-GFP; D = (GAGAGS)$_3$-GFP; E = (GAGAGS)$_4$-GFP; F = (GAGAGS)$_5$-GFP; G = (GAGAGS)$_6$-GFP; and H = (GAGAGS)$_7$-GFP respectively

Table 9: Concentration of (GAGAGS)$_n$-GFP peptide by Bradford assay

Peptides	Concentration (mg/ml)
GFP	8.28
GAGAGS-GFP	7.05
(GAGAGS)$_2$-GFP	7.89
(GAGAGS)$_3$-GFP	8.04
(GAGAGS)$_4$-GFP	5.56
(GAGAGS)$_5$-GFP	6.69
(GAGAGS)$_6$-GFP	6.83
(GAGAGS)$_7$-GFP	9.32

4.3 Formation of SF/(GAGAGS)$_n$-GFP hydrogel

Determination time of SF hydrogel was performed in 96-well microplate that each well composed of vortexed SF solution and (GAGAGS)$_n$-GFP at the same volume and final concentration adjusted to 4% w/v of SF solution and 100 μg/ml of (GAGAGS)$_n$-GFP peptides. The solution was incubated at 37 $^{\circ}$C and measured absorbance at 550 nm wavelength every 2 hours. The gelation time results were showed in fig 7 and table 10. The SF solution with (GAGAGS)$_5$-GFP, (GAGAGS)$_6$-GFP, and (GAGAGS)$_7$-GFP formed hydrogels within 55 hours. After 58 hours of experiment time, the SF solution with (GAGAGS)$_2$-GFP, (GAGAGS)$_3$-GFP and (GAGAGS)$_4$-GFP formed hydrogel. In addition, the condition of SF with GAGAGS-GFP and untagged GFP formed hydrogel within 72 hours. Finally, the SF solution alone showed the latest of forming hydrogel. The results in this experiment concluded that increasing the repeat unit of SF (GAGAGS) could reduce the gelation time of SF hydrogel.
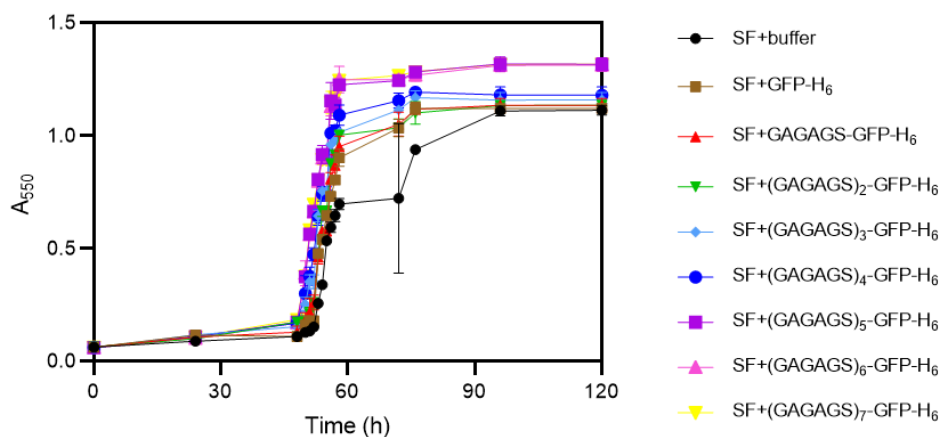
Figure 7: The gelation time of SF hydrogel in different of (GAGAGS)$_n$-GFP peptides.

Table 10: The gelation time point of SF solution with (GAGAGS)$_n$-GFP peptides formed hydrogel.

Conditions	Gelation time point of SF hydrogel
SF+buffer	96 hours
SF+GFP	72 hours
SF+GAGAGS	72 hours
SF+(GAGAGS)$_2$-GFP	58 hours
SF+(GAGAGS)$_3$-GFP	58 hours
SF+(GAGAGS)$_4$-GFP	56 hours
SF+(GAGAGS)$_5$-GFP	55 hours
SF+(GAGAGS)$_6$-GFP	55 hours
SF+(GAGAGS)$_7$-GFP	55 hours

Decreasing of

gelation time

4.4. *In vitro* release of GFP from SF hydrogel

To explore the release profile of GFP from SF hydrogel, the GFP release was determined by its absorbance at 395 nm light. Interestingly, , the absorbance of GFP that release from SF hydrogel assembly with GFP, GAGAGS-GFP, and $(GAAGS)_2$-GFP occurred the fastest equilibrium value with 10 minutes and this value stable until the end of experiment (Fig 8a). While the GFP that release from SF/$(GAGAGS)_3$-GFPand SF/$(GAGAGS)_4$-GFP hydrogel showed the highest of absorbance value and reached the equilibrium within 80 minutes still equilibrated until the end of testing (Fig 8a). Interestingly, the SF hydrogel with $(GAGAGS)_5$-GFP, $(GAGAGS)_6$-GFP, and $(GAGAGS)_7$-GFP showed gradually increase of GFP absorbance and occurred the highest value and reached the equilibrium within at 48 hours (2,880 minutes), 72 hours (4,320 minutes), and 96 hours (5,760 minutes) respectively (Fig 8b).
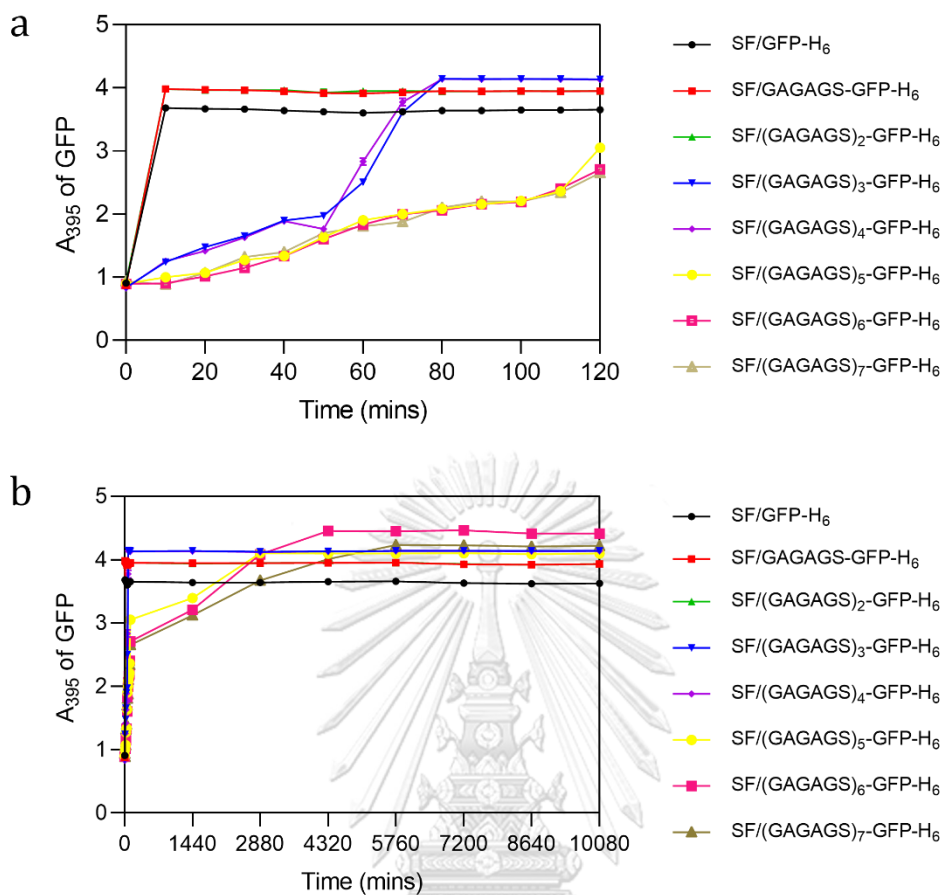
Figure 8: In vitro release profiles of the recombinant GFP from the SF hydrogels

4.5 *In vitro* enzymatic degradation of SF/(GAGAGS)$_n$-GFP hydrogel

To explore the effect of repeating unit on degradation of SF hydrogel, the hydrogels were submerged in PBS buffer that containing 1 U/ml protease XIV. Generally, the SF hydrogel was not easily degraded in normal environment without specific enzyme because it had high beta-sheet content. After 30 days, the SF hydrogel containing (GAGAGS)$_5$-GFP, (GAGAGS)$_6$-GFP, and (GAGAGS)$_7$-GFP remained the

weight of hydrogel cube upper 50% of gel weight compare in first day (Fig 9). In

contrast, the condition had GFP, GAGAGS-GFP, (GAGAGS)$_2$-GFP showed the lowest of

weight SF hydrogel (Fig 9). And the sample of (GAGAGS)$_3$-GFP, and (GAGAGS)$_4$-GFP

hydrogel remained weight of SF hydrogel around 20-30% that showed in Fig 9. These

results indicated that the degradation rate of SF hydrogels contained varying of

repeat unit depended on the number of silk fibroin repeat unit(s).
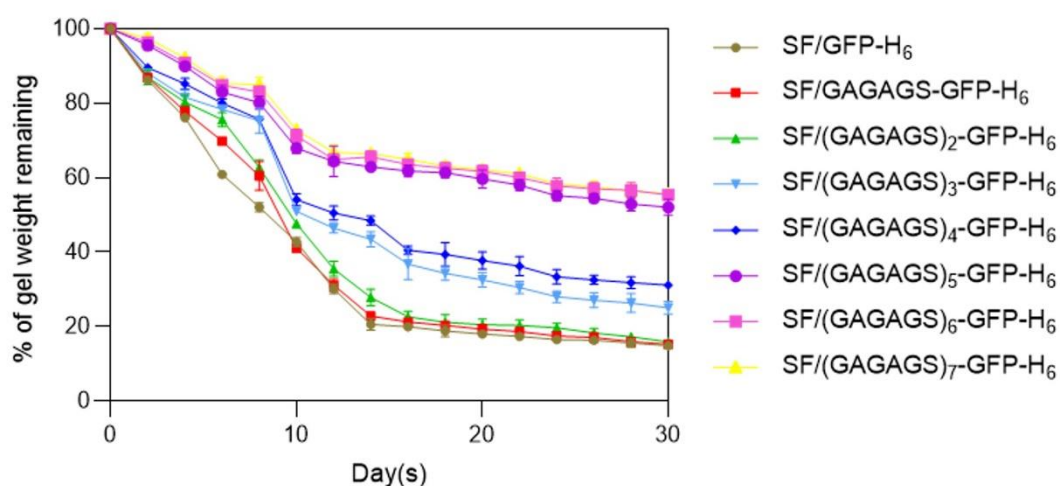
Figure 9: SF hydrogel degradation profile was showed the percentage of
SF/(GAGAGS)$_n$-GFP hydrogels weight remaining that incubated in protease XIV
(1 U/ml) solution at 37 °C around 30 days.

4.6 Characterization of SF/(GAGAGS)$_n$-GFP lyophilized hydrogel

SF protein secondary structure was analyzed by Fourier Transform Infrared spectroscopy (FTIR) and X-ray diffraction (XRD). FTIR was a common tool for analysis the molecular structure conformation. Normally, SF secondary structure had four main structures; random coil, alpha-helix, beta-sheet, and beta turn especially beta-sheet and random coil structure were a major structure in SF protein. The spectrum wavenumber of SF hydrogel mixed GFP, GAGAGS-GFP, (GAGAGS)$_3$-GFP, and (GAGAGS)$_6$-GFP demonstrated peak slightly shifted the amide I absorption peaks from 1650 cm^{-1} (random coils) toward 1630 cm^{-1} (β-sheet). However, the shift in the amide II absorption was unclear (Fig 10). This result suggested that longer repeating unit (GAGAGS) induced more crystalline β-sheet formation.
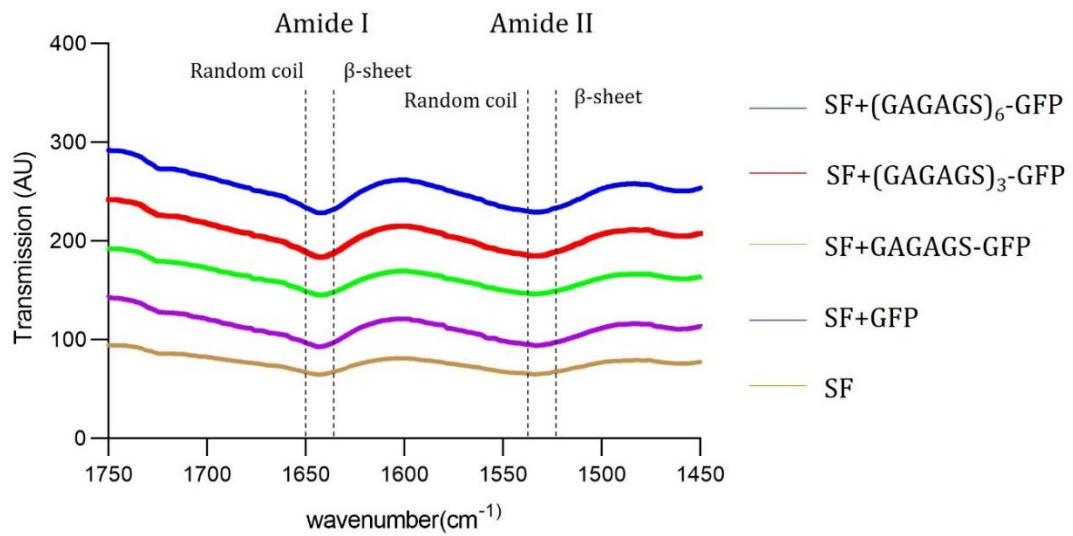
Figure 10: Fourier-transform infrared spectroscopy (FTIR) spectra of SF hydrogel around the absorption bands of amide I and amide II

Another tool for analysis SF protein structure was X-ray diffraction (XRD). The crystalline of SF protein mainly occurred peak around 19.5 – 22.5°. The spectrum XRD results showed peak at 22.36° for SF, 19.62° for SF+GFP, 21.19° for SF+GAGAGS-GFP, 20.76° for SF+(GAGAGS)$_3$-GFP and 21.07° for SF+(GAGAGS)$_6$ (Fig 11). These results showed that the peak in XRD spectra represented crystalline region or beta-sheet

structure   were   increased   with   increasing   number   of   repeat   units
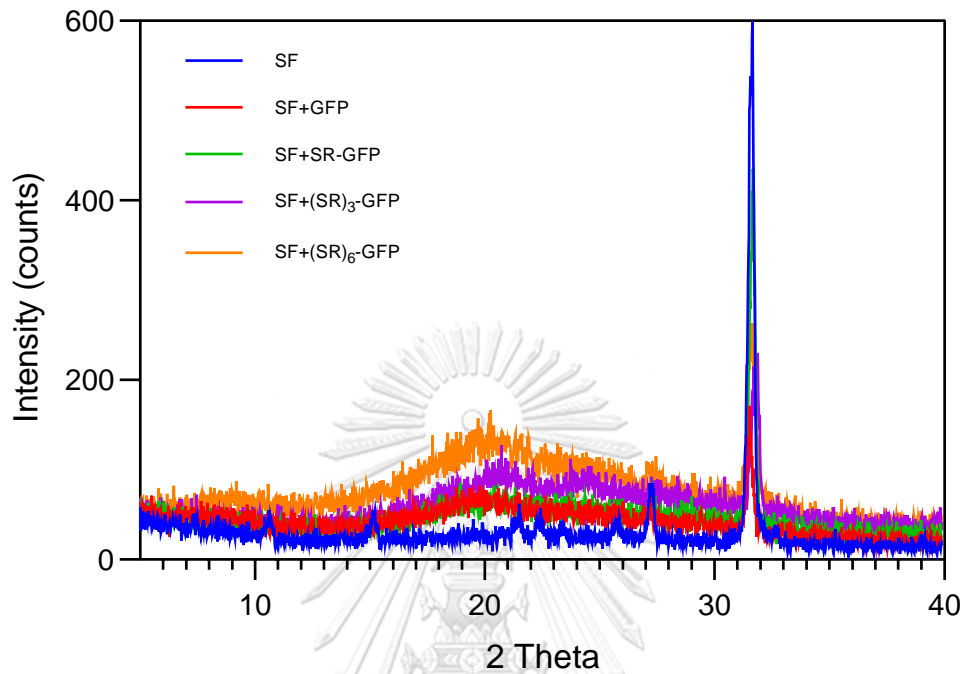
Figure 11: X-ray diffraction (XRD) pattern from 5° – 40° of SF hydrogel

4.7 Construction of (GAGAGS)$_n$-tagged bFGF expression plasmids

The DNA sequences coding for the SF repeating unit (GAGAGS) of zero and six

repeat unit were fused to the DNA sequence of basic fibroblast growth factor (bFGF).

TEV-(GAGAGS)$_n$-bFGF fragments was amplify by polymerase chain reaction (PCR).

Detection of the PCR product was analyzed by agarose gel electrophoresis (Fig 12)

that bFGF and TEV-bFGF PCR product showed size band between 400 to 600 bp.

After PCR product purification, pET28b and pMALC5x were cut with *NcoI* and *HindIII* restriction enzyme and the PCR product cloned into by ligation. Then, the ligation reaction was transformed to *E. coli* DH10β. To confirm the cloning result, the transformed *E. coli* DH10β were checked that the TEV-(GAGAGS)$_n$-GFP fragments were constructed into the expression plasmid by colony PCR. The positive construction plasmids were extracted and confirmed by nucleic acid sequencing. The nucleic acid sequencing of bFGF fragment in pET28b plasmid and TEV-bFGF fragment in pMALc5x were shown in table 11 and the amino acid sequences that translated from the nucleic acid sequencing was shown in table 12.

Figure 12: TEV-bFGF in lane A and bfgf in lane B PCR product in 1% agarose gel.

pMALc5x-TEV-(GAGAGS)$_6$-bFGF plasmid was synthesized and purchased from

Biomatik company. The DNA sequences of bFGF-(GAGAGS)$_6$-bFGF fragment was
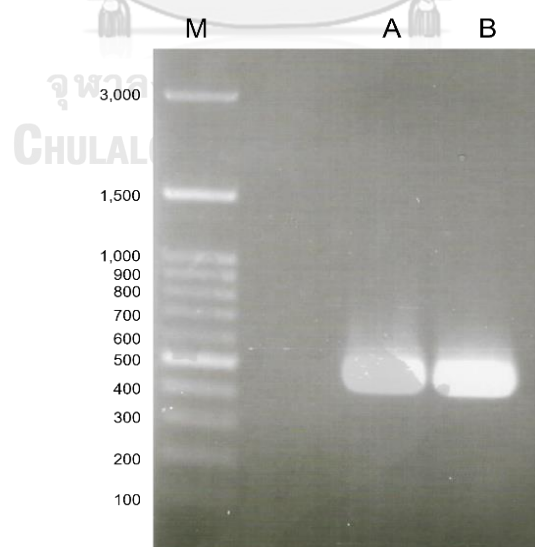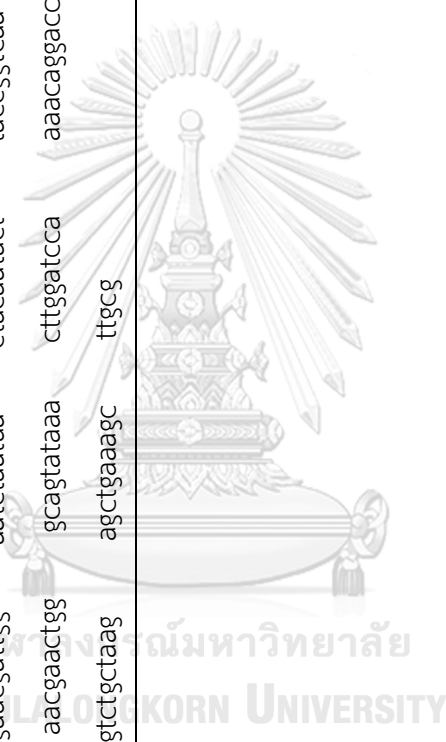
showed in table 11 and the amino acid sequences that was translated from the

nucleic acid sequencing was shown in table 12.

Table 11: DNA sequence of bFGF, TEV-bFGF, and TEV-(GAGAGS)$_6$-bFGF fragment

Fragment	DNA sequence						
bFGF	ataccatggc	agccgggagc	atcaccacgc	tgcccgcctt	gcccgaggat	ggcggcagcg	gcgccttccc
	gcccggccac	ttcaaggacc	ccaagcggct	gtactgcaaa	aacgggggct	tcttcctgcg	catccacccc
	gacggccgag	ttgacggggt	ccgggagaag	agcgaccctc	acatcaagct	acaacttcaa	gcagaagaga
	gaggagttgt	gtcttatcaaa	ggagtgtgtg	ctaaccgtta	cctggctatg	aaggaagatg	gaagattact
	ggcttctaaa	tgtgttacgg	atgagtgttt	cttttttgaa	cgattggaat	ctaataacta	caatacttac
	cggtcaagga	aatacaccag	ttggtatgtg	gcactgaaac	gcactgggca	gtataaactt	ggatccaaaa
	caggacctgg	gcagaaagct	atactttttc	ttccaatgtc	tgctaagagc	tgaaagcttg	cg
TEV-bFGF	ataccatggg	acaccaccac	caccaccacg	gttccgaaaa	cctgtatttt	cagggggtg	ctggctctgg
	ttcatcaggt	atggcagccg	ggagcatcac	cacgcgtccc	gccttgcccg	aggatggcgg	cagcggcgcc
	ttcccgcccg	gccacttcaa	ggaccccaag	gcaaaaacgg	gggcttcttc	gggcttcttc	ctgcgcatcc
	accccgacgg	ccgagttgac	ggggtccggg	agaagagcga	ccctcacatc	aagctacaac	ttcaagcaga
	agagagagga	gttgtgtcta	tcaaaggagt	gtgtgctaac	cgttacctgg	ctatgaagga	agatggaaga
	ttactggctt	ctaaatgtgt	tacggatgag	tgtttctttt	ttgaacgatt	ggaatctaat	aactacaata
	cttaccggtc	aaggaaatac	accagttggt	atgtggcact	gaaacgaact	gggcagtata	aacttggatc
	caaaacagga	cctgggcaga	aagctatact	ttttcttcca	atgtctgcta	agagctgaaa	gcttgcg
TEV- (GAGAGS)$_6$- bFGF	ataccatggg	acaccaccac	caccaccacg	gttccgaaaa	cctgtatttt	cagggcggtg	ctggagccgg
	ctctggggcc	ggcgctggct	cgggtgctgg	agccgccgcg	ggggccggcg	ctggctcggg	ggcaggagcc

gggtctggag	caggagcagg	aagtggtgct	ggctctggtt	catcaggtat	ggcagccggg	agcatcacca
cgctgcccgc	cttgcccgag	gatggcggca	gcggcgcctt	cccgcccggc	cacttcaagg	accccaagcg
gctgtactgc	aaaaacgggg	gcttcttcct	gcgcatccac	cccgacggcc	gagttgacgg	ggtccgggag
aagagcgacc	ctcacatcaa	gctacaactt	caagcagaag	agagaggagt	tgtgtctatc	aaaggagtgt
gtgctaaccg	ttacctggct	atgaaggaag	atggaagatt	actggcttct	aaatgtgtta	cggatgagtg
tttctttttt	gaacgattgg	aatctaataa	ctacaatact	taccggtcaa	ggaaatacac	cagttggtat
gtggcactga	aacgaactgg	gcagtataaa	cttggatcca	aaacaggacc	tgggcagaaa	gctatacttt
ttcttccaat	gtctgctaag	agctgaaagc	ttgcg			

Table 12: Amino acid sequence of bFGF, TEV-bFGF and TEV-(GAGAGS)₆-bFGF peptides.

Peptide	Amino acid sequence					
bFGF	MAAGSITTLP	ALPEDGGSGA	FPPGHFKDPK	RLYCKNGGFF	LRIHPDGRVD	GVREKSDPHI
	KLQLQAEERG	VVSIKGVCAN	RYLAMKEDGR	LLASKCVTDE	CFFFERLESN	NYNTYRSRKY
	TSWYVALKRT	GQYKLGSKTG	PGQKAILFLP	MSAKS		
H₆-TEV-bFGF	MGHHHHHHGS	ENLYFQGGAG	SGSSGMAAGS	ITTLPALPED	GGSGAFPPGH	FKDPKRLYCK
	NGGFFLRIHP	DGRVDGVREK	SDPHIKLQLQ	AEERGVVSIK	GVCANRYLAM	KEDGRLLASK
	CVTDECFFFE	RLESNNYNTY	RSRKYTSWYV	ALKRTGQYKL	GSKTGPGQKA	ILFLPMSAKS
H₆-TEV-(GAGAGS)₆-bFGF	MGHHHHHHGS	ENLYFQGGAG	AGSGAGAGSG	AGAGSGAGAG	SGAGAGSGAG	AGSGAGSGSS
	GMAAGSITTL	PALPEDGGSG	AFPPGHFKDP	KRLYCKNGGF	FLRIHPDGRV	DGVREKSDPH
	IKLQLQAEER	GVVSIKGVCA	NRYLAMKEDG	RLLASKCVTD	ECFFFERLES	NNYNTYRSRK
	YTSWYVALKR	TGQYKLGSKT	GPGQKAILFL	PMSAKS		

4.8 bFGF, H_6-TEV-bFGF and H_6-TEV-(GAGAGS)$_6$-bFGF expression and purification

4.8.1 bFGF expression and purification

bFGF expression using *E. coli* was optimized for an appropriate IPTG concentration. The result, shown in Fig 13, indicated that bFGF expressed as high amount of soluble protein. Even though the bFGF expression can be detected without inducing by IPTG, IPTG (0.1 μM) was used in large scale production.
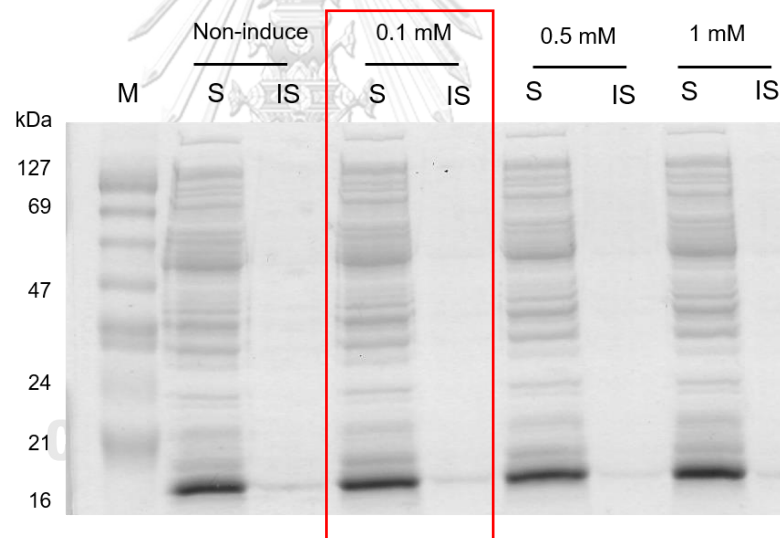
Figure 13: The optimization of IPTG varying on IPTG concentration. M = protein marker, S = soluble protein (from supernatant sample), IS = insoluble protein (from cell debris sample)

After bFGF large scale production, soluble protein was extracted and kept the supernatant for purify step. The 0.25% of PEI solution was added into supernatant for precipitated contaminant protein Fig 14.
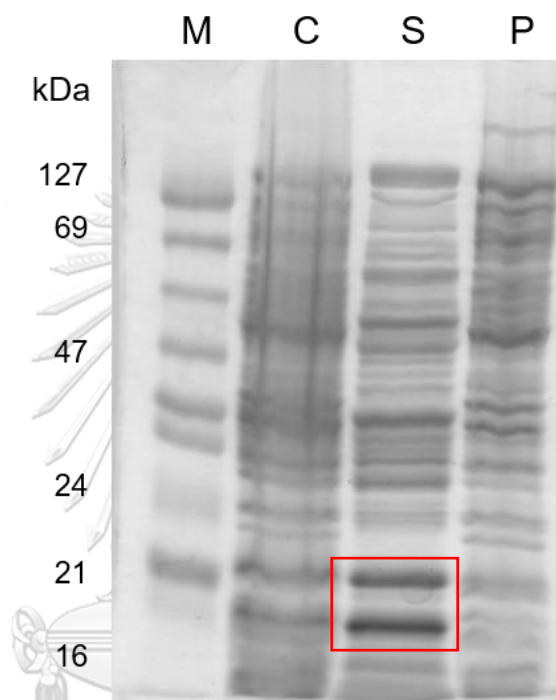
Figure 14: The PEI precipitate purification in 0.25% v/v concentration.M = protein marker, C = crude protein, S = soluble protein, P = precipitated protein; The SDS-PAGE result shows the bFGF band around 16-21 kDa in soluble fraction that show the higher intensity band.

After precipitation, the supernatant was collected and precipitated again with 2 M of ammonium sulfate (($NH_4)_2SO_4$)) to precipitate impurity component. Then

supernatant was purified by hydrophobic interaction chromatography. The bFGF

peptide can bind to hydrophobic column and eluted with gradient step of $(NH_4)_2SO_4$

from 2 M concentration to 0 M concentration of $(NH_4)_2SO_4$. The hydrophobic result is

shown in Fig 15. In this case, the E_1-E_3 of eluted bFGF were selected.
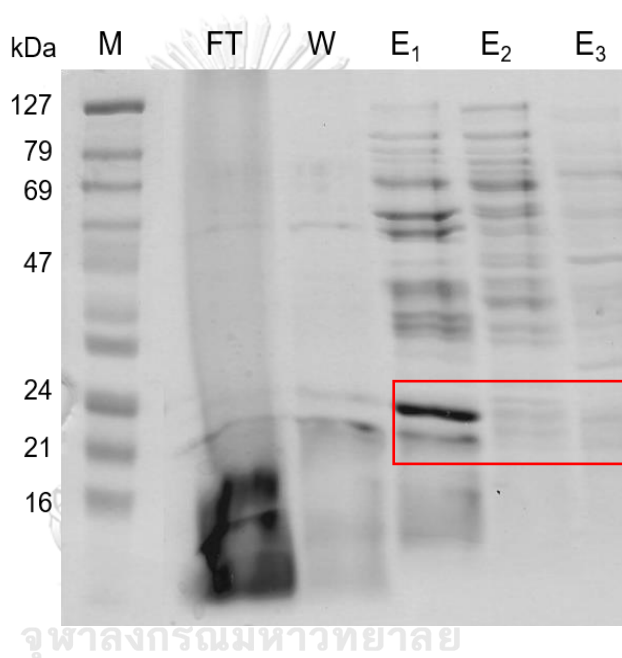
Figure 15: The purification of bFGF peptide by hydrophobic column.M = protein marker, FT = protein in 2 M $(NH_4)SO_4$, W = wash fraction with 2 M $(NH_4)SO_4$, E_1 = wash fraction with 1 M $(NH_4)SO_4$, E_2 = wash fraction with 0.5 M $(NH_4)SO_4$, E_3 = wash fraction with 0 M $(NH_4)SO_4$

After gradient elution, the bFGF was purified with ion-exchange

chromatography. Before purified this peptide, the peptide was dialyzed in 20 mM

Tris, pH 7.5 for remove $(NH_4)_2SO_4$). Then, the peptide purified with SP column and

the peptide purity was shown in Fig 16. In this case, the F_{29}-E_1 fractions of eluted

bFGF were selected. After concentration using centrifugal filter, the peptide

concentration was determined using A_{280} following by Beer's law and shown the

concentration in table 16

Figure 16: The bFGF purification by ion-exchange chromatography.M = protein marker, IP = protein input solution, FT = protein unbonded solution, F_1-F_{29} = bFGF eluted fraction of 0 mM – 1 M NaCl concentration, E_1 = eluted fraction in 1 M NaCl

4.8.2 H_6-TEV-bFGF and H_6-TEV-(GAGAGS)$_6$-bFGF expression

H_6-TEV-bFGF and H_6-TEV-(GAGAGS)$_6$-bFGF expression using *E. coli* were optimized the IPTG concentration. The results, shown in Fig 17, indicated that H_6-TEV-bFGF (Fig 17a) and H_6-TEV-(GAGAGS)$_6$-bFGF (Fig 17b) expressed as high amount of soluble protein. Even though the (His)$_6$-TEV-bFGF and H_6-TEV-(GAGAGS)$_6$-bFGF expression can be detected without inducing by IPTG, IPTG (0.1 μM) was used in large scale production.
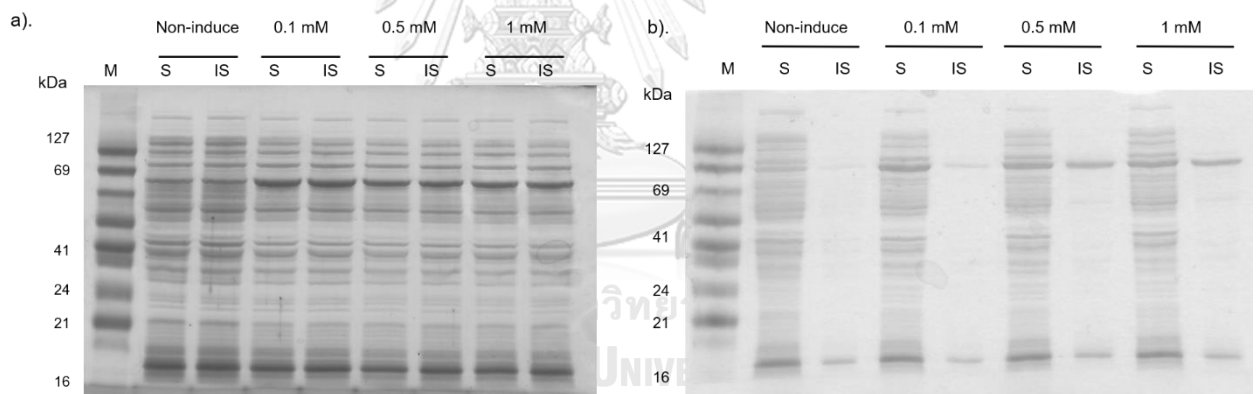
Figure 17: The optimization of IPTG varying on IPTG concentration.M = protein marker, S = soluble protein (from supernatant sample), IS = insoluble protein (from cell debris sample). a). IPTG concentration optimization for H_6-TEV-bFGF expression and b). IPTG concentration optimization for H_6-TEV(GAGAGS)$_6$-bFGF.

4.8.2.1 H_6-TEV-bFGF purification

After H_6-TEV-bFGF large scale production, soluble protein was extracted and

kept the supernatant for purify step. H_6-TEV-bFGF was purified using Ni-NTA agarose

column. The result of the first purification step was detected by Coomassie blue

staining Fig. 18. Then, TEV protease was added to MBP-H_6-TEV-bFGF fraction for

digested at TEV site. After that, the digestive peptide was purified again with Ni-NTA

agarose column (Fig 18).

Figure 18: The histidine tag-containing TEV-bFGF purification.M = protein marker.
The 1st Ni-column; C = protein input solution, FT_1 = protein unbonded solution, W_1 =
protein in 25 mM imidazole-containing buffer washed fraction, E_1 = MBP-H_6-TEV-
bFGF in 250 imidazole buffer. T = TEV protease cut check fraction. The 2nd Ni-
column; FT_2 = bFGF, W_2 = bFGF in 25 mM imidazole-containing buffer washed
fraction, E_2 = MBP-H_6 in 250 imidazole buffer

The flowthrough fraction of bFGF peptide in 2nd Ni-NTA agarose column was

purified with ion-exchange chromatography with SP column for remove TEV

protease. The peptide purification result is shown in Fig 19. In this case, the E4-E7

fractions of eluted bFGF were selected. After concentration using centrifugal filter,

the peptide concentration was determined using A$_{280}$ following by Beer's law and
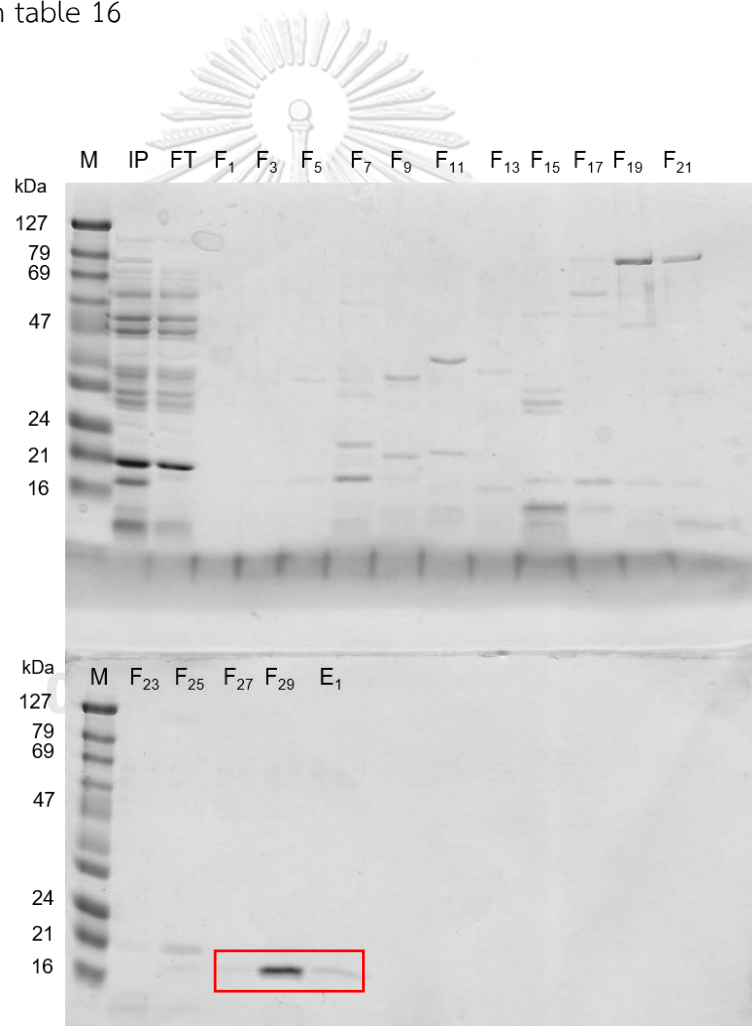
shown the concentration in table 16.

Figure 19: The bFGF purification by ion-exchange chromatography.M = protein marker, FT = protein unbonded solution, E$_1$-E$_8$ = eluted protein fraction from 0 mM – 1 M of NaCl in 20 mM Tris, pH 7.5 buffer.

4.8.2.2 H_6-TEV-(GAGAGS)$_6$-bFGF purification

After H_6-TEV-(GAGAGS)$_6$-bFGF large scale production, soluble protein was extracted and the supernatant kept for purification. H_6-TEV-(GAGAGS)$_6$-bFGF was purified using Ni-NTA agarose column. Then, TEV protease was added to MBP-H_6-TEV-(GAGAGS)$_6$-bFGF fraction for digested at TEV site. After that, the digestive peptide was purified again with Ni-NTA agarose column (Fig 20).
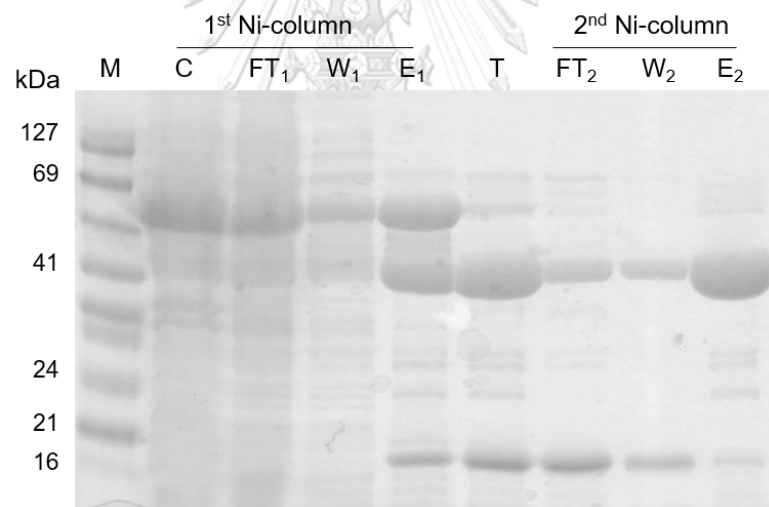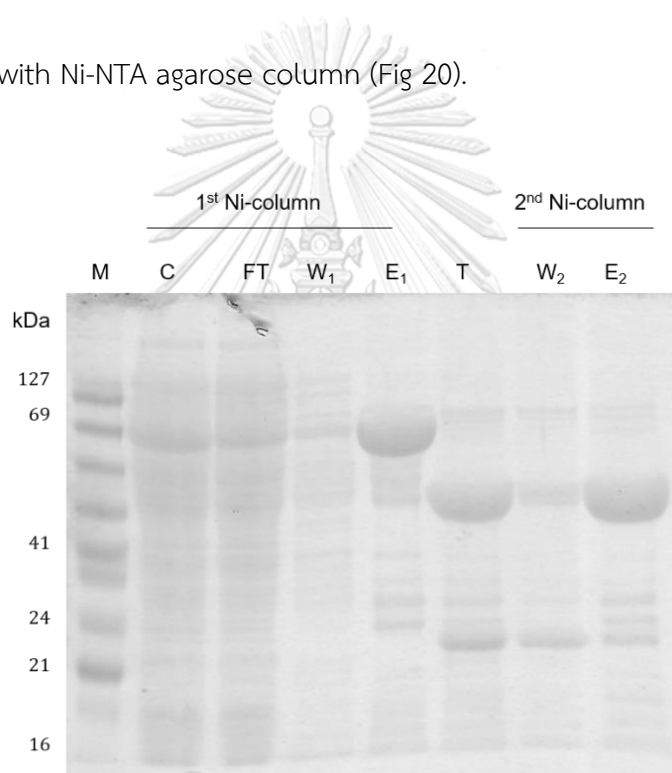

Figure 20:The histidine tag-containing TEV-(GAGAGS)$_6$-bFGF purification.M = protein marker. The 1st Ni-column; C = protein input solution, FT = protein unbonded solution, W_1 = protein in 25 mM imidazole-containing buffer washed fraction, E_1 = MBP-H_6-TEV-(GAGAGS)$_6$-bFGF in 250 imidazole buffer. T = TEV protease cut check fraction. The 2nd Ni-column; W_2 = (GAGAGS)$_6$-bFGF in 25 mM imidazole-containing buffer washed fraction, E_2 = MBP-H_6 in 250 imidazole buffer.

The flow-through fraction of H6-TEV-(GAGAGS)6-bFGF peptide from the 2nd Ni-

NTA agarose column purification was further purified with ion-exchange

chromatography. The peptide purification result is shown in Fig 21. In this case, the

E3-E5 fractions of eluted H6-TEV-(GAGAGS)6-bFGF were selected. After concentration

using centrifugal filter, the peptide concentration was determined using A_{280} following

by Beer's law and shown the concentration in table 16.
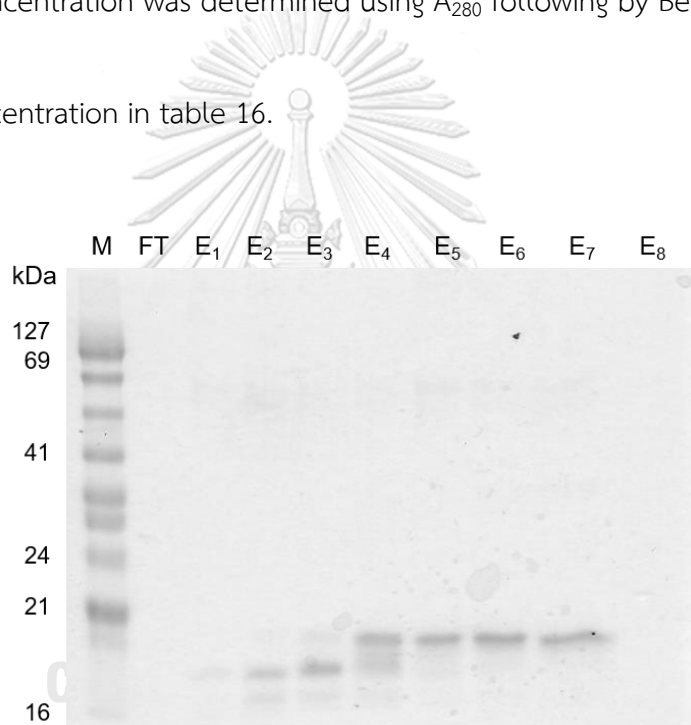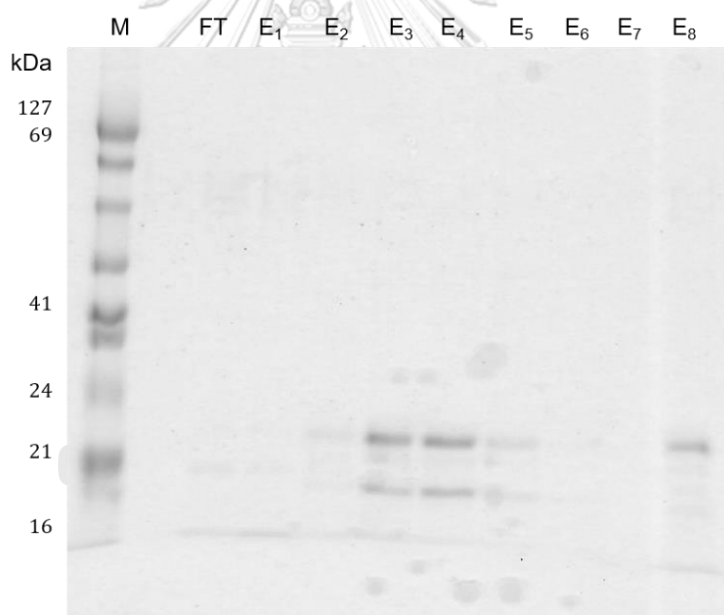

Figure 21: The (GAGAGS)$_6$-bFGF purification by ion-exchange chromatography. M =

protein marker, FT = protein unbonded solution, E$_1$-E$_8$ = eluted protein fraction from

0 mM − 1 M of NaCl in 20 mM Tris, pH 7.5 buffer.

Table 13: The concentration of bFGF recombinant peptide

Peptide	A_{280}	ε (M^{-1} cm^{-1})	Concentration (mg/ml)
bFGF from pET28b	0.604	15,930	0.65
bFGF from pMALc5x	0.979	15,930	1.10
(GAGAGS)$_6$-bFGF from pMALc5x	1.02	15,930	1.29

4.9. Application of bFGF, bFGF from pMALc5x and (GAGAGS)$_6$-bFGF peptide on tissue engineering

4.9.1 Optimization of bFGF, bFGF from pMALc5x and (GAGAGS)$_6$-bFGF concentration for cell proliferation.

Before applied these peptides on tissue engineering, The optimization of peptides concentration for cell survival was determined. NIH-3T3 proliferation (10^3 cells/ml) was determined by various concentration of peptides from 0 – 100 ng/ml. After 24 hour of cell culture, MTT assay was performed for elucidate the cell proliferation. The result, shown in Fig 22, explained that 100 ng/ml of all peptides had the highest of absorbance of 570 nm light and lower absorption at 1 and 10

ng/ml peptide concentration. The absorption value in these results can elucidated

that all peptides can induced NIH-3T3 proliferation and showed the function of

native form.
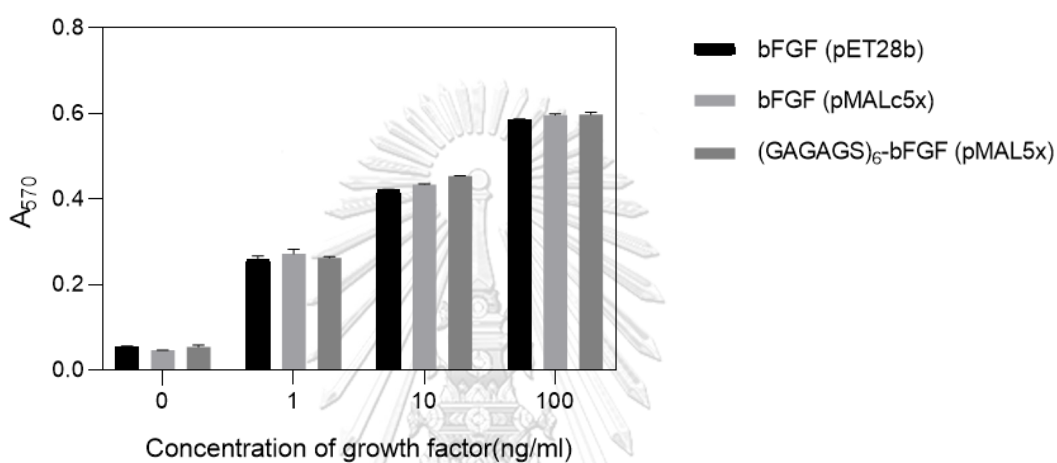
Figure 22: Concentration optimization of three types bFGF peptide on NIH-3T3
proliferation detected by MTT assay.

4.9.2 *In vitro* bFGF release determined by cell proliferation.

To explore the repeat unit of SF on CRS in tissue engineering application,

Production of SF, SF/bFGF, SF/bFGF$_{(pMALC5x)}$, and SF/(GAGAGS)$_6$-bFGF hydrogel were

performed and cut these hydrogels into cubic shape. After that, NIH-3T3 were

cultured in 6-well plates containing 0.1% FBS culture media and each well had four

hydrogels cubic that had 1 ng/ml of peptide. The metabolic activity of cell culture

with SF/bFGF and SF/bFGF$_{(pMALC5x)}$ rapidly increased of A$_{570}$ in 8 days and stable over the time course of 16 days culture periods. Interestingly, the metabolic activity value of cell culture with SF/(GAGAGS)$_6$-bFGF hydrogel gradually increased in day 10 and stable over the time course of culture periods Fig 23. These results illustrated that increasing repeat unit of SF could control bFGF release in first linear and sustained release for support NIH-3T3 proliferation.
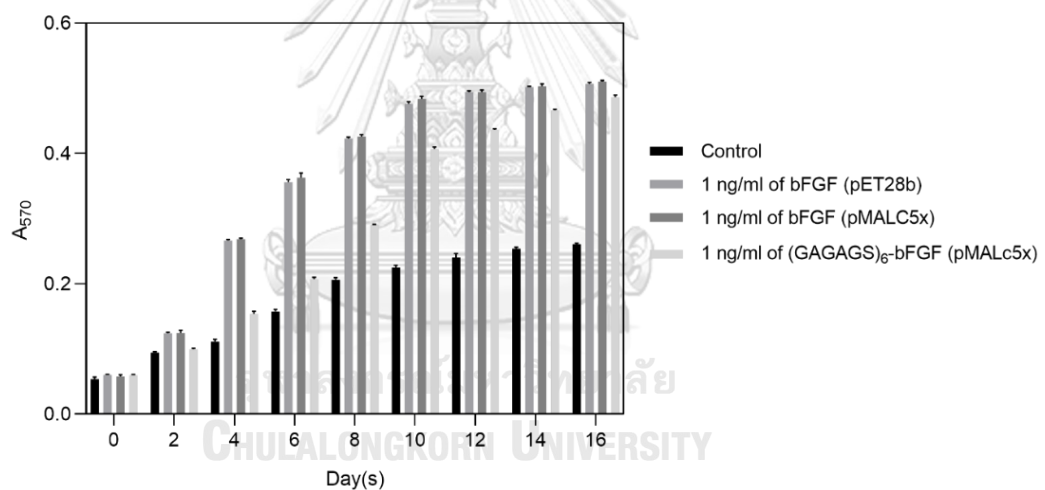
Figure 23: In vitro of bFGF release from SF hydrogel on NIH-3T3 proliferation detected by cellular activity assay (MTT)

CHARPTER V

DISCUSSION AND CONCLUSION

Many medical drugs are conventional ("free") drug delivery systems (DDS) for medical treatment, but these systems are hardly controlled processes because the drug release rate is difficult to manage. These effects can cause many disadvantages such as poor solubility, tissue damage, unfavorable pharmacokinetics, and lack of selectivity for target tissue. Control release system (CRS) is an important class of DDS for improving drug release (Tibbitt et al., 2016). Nowadays, the natural polymer CRS material has been developed and designed as a vehicle not only for carrier a drug and sustain release at desirable concentration and correct target but also there can protect the biological function of the protein drug (Tong et al., 2020). Silk fibroin (SF) is the most popular natural polymer used in CRS (Wenk et al., 2011). SF had various good properties such as biocompatibility, mechanical strength, and biocompatibility and showed an excellent choice for the delivery of bioactive molecules (Koh et al., 2015). In the past, there have many studies that used conventional SF-based

hydrogel-controlled drug release and maintained bioactive compound activity, but

these molecules release from SF hydrogel in a non-linear manner (de Moraes et al.,

2015). To solve these problems, SF/copolymer composite hydrogel was used to

improve the release profile because the hydrophobic and hydrophilic of copolymer

could help to sustain drug release, but this system is non-linear drug release manner

(Zhong et al., 2015).

The primary structure of SF protein contains two polypeptide chains

hydrophobic heavy chain and a hydrophilic light chain that has a molecular weight of

around 200 – 350 kDa. The amino acid sequence of the hydrophobic heavy chain has

a repetitive block and the sequence GAGAGS repeat unit shows the highest repetitive

unit in this chain. The repetitive unit of hydrophobic block folds and form β-sheet

crystalline structure via hydrophobic bonds, van der Waals force, and hydrophobic

interaction (Zhou et al., 2001). These crystalline structures of SF can help control

drug release profiles. From the knowledge and advantage properties of repetitive unit

sequence in natural protein, several researchers have tried to synthesize repeating

units of protein that mimic the known amino acid sequence in natural protein

structure. For example, Collagen mimetic peptide or CMPs has synthesized 7 Gly-X-Y

units and coupled to a growth factor that mimics the natural collagen. CMPs can

adhere to loops or interrupt the triple helix of natural collagen and sustain the

release of growth factors from mimic CMPs in wound healing (Chattopadhyay et al.,

2012). Another research was on the recombinant spider silk protein eADF4 (C16) that

mimicked the 16 repeating unit amino acid sequence of spider silk protein. The

mimic spider silk can be prepared into various forms of drug carrier and has shown

that spider silk was the potential to sustain the drug release system (Spiess et al.,

2010).

In this study, the repeating unit of silk fibroin (GAGAGS) fused with GFP amino

acid sequence was synthesized from zero of GAGAGS to seven of GAGAGS and

purified into pure peptides by chromatography methods. These recombinant

peptides had the function that was detected by its green fluorescent color of GFP

peptide shown in Fig 4.2b. The effect of the repeat unit of SF on CRS was

determined by in vitro release. The release profile of GFP from SF hydrogel showed

GFP release accumulation detected by its GFP absorbance at 395 nm light (Prasher et

al., 1992). A release accumulation at equilibrium of GFP release was detected at 10

minutes. The release rate of GFP was subsequently decreased by increasing the

GAGAGS unit of SF. Surprisingly, the highest [(GAGAGS)$_7$-GFP] in SF hydrogel showed

the slowest release rate and GFP accumulation at equilibrium occurred in 96 hours.

Not only increasing the GAGAGS unit of SF can reduce the burst release effect and

control the protein drug release profile but also all repeat units can maintain and

sustain release for up to one week. The result can explain that the recombinant

(GAGAGS)$_n$-GFP peptides had hydrophobic interaction with the repeat unit of the SF

hydrogel scaffold. This evidence showed that increasing the GAGAGS unit can

increase interaction fore between GAGAGS mimic peptide and nature GAGAGS

scaffold that controlled the release rate and sustained release profile (Koh et al.,

2015). The effect of (GAGAGS)$_n$-GFP was showed in the gelation time of SF hydrogel

(Fig. 4.3). The larger GAGAGS units can induce gel formation or reduced the gelation

time of SF hydrogel. The hydrogel formation was dealt with secondary structure

change of SF protein. The SF structure in SF solution changed from a disordered

stated to β-sheet conformation in a hydrogel state (Matsumoto et al., 2006). This

result explained that the higher GAGAGS unit can change the structure from disorder to crystalline is faster and depend on the increase of hydrophobic fore too. In addition, the increasing unit of $(GAGAGS)_n$-GFP can reduce the degradation rate of SF hydrogel. The remaining SF/$(GAGAGS)_n$ = 5,6,7-GFP hydrogel weight showed an upper 50% at the end of the experiment (Day 30). This result can exhibit that the GFP release profile from SF hydrogel may depend on degradation mechanism and the SF hydrogel with a larger unit of SF can help to prolong the scaffold for tissue regeneration (Lu et al., 2011).

To find the reason about the GFP release process, the structure analysis of repeating unit tagged GFP peptide loaded hydrogel by FTIR and XRD revealed that the repeat unit-tagged GFP peptide can increase the crystalline β-sheet fraction in the hydrogel. Therefore, the in vitro release of GFP was not only dependent on the secondary structure of SF but this process also involves in intermolecular force between repeat unit of mimicked peptide and SR unit in SF hydrogel.

Finally, the benefit of repeat unit SF was applied in tissue engineering (Kasoju & Bora, 2012). The recombinant of bFGF and $(GAGAGS)_6$-bFGF was produced. These recombinant peptides had function because their induced cell line proliferation detected by metabolic activity (MTT assay) (Bikfalvi et al., 1997). Subsequently, the application of recombinant peptide on cellular function was performed. The NIH-3T3 was slightly grow when incubated with $(SR)_6$-bFGF while was rapidly proliferated with bFGF. The larger of SR tagged growth factor could be reduce the bust release effect and controlled release of bFGF from scaffold. This advantage can be applied in tissue engineering for medical proposes.

Conclusions

The repeating unit of SF tagged protein drug mimic peptide does not alter the function of the tagged protein. In addition, increasing the repeat unit can reduced the gel formation. The tagged protein can be attached into SF hydrogel possibility by interacting with crystalline β-sheets structure. The interaction between repeating unit mimic peptide and SF scaffold leads to the sustained release of the protein drug and

slower degradation of the hydrogel. Moreover, increasing the repeat unit-tagged

growth factor can control the growth factor release profile. The result in this

dissertation provides evidence that genetic engineering of the sequence and length

of repeat unit mimic peptide allows control the release rate and degradation rate to

use in diverse application.

APPENDIX

Culture media for bacteria

LB

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Water	1L
(Agar)	(15 g)

SOB
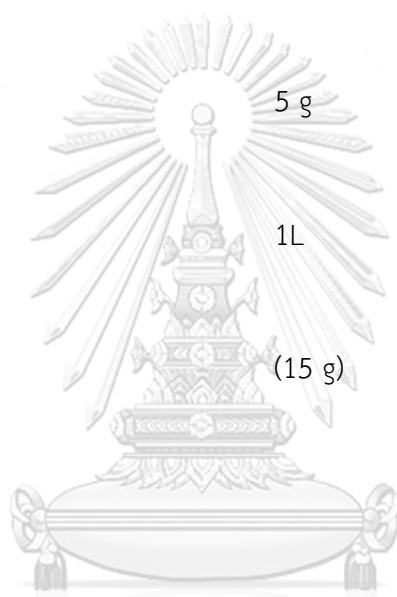
Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
1 M KCl	2.5 ml

1 M MgCl$_2$	10 ml
1 M MgSO$_4$	10 ml
Water	To 1 L

(SOC = SOB + 20 mM Glucose)

Terrific broth

Tryptone	12 g
Yeast extract	24 g
Glycerol	4 ml
Water	To 1 L

CCMB80 buffer pH 6.4

KOAc	0.981 g
$CaCl_2$	11.8 g
$MgCl_2$	2.03 g
Glycerol	100 ml
$MnCl_2$ (added later)	1.98 g
Water	To 1 L

TSB buffer

Tryptone	10 g
Yeast extract	5 g
NaCl	5.84 g
PEG3350	100 g

DMSO	50 ml

MgCl$_2$	0.95 g

MgSO$_4$	1.20 g

Phosphate buffer

KH$_2$PO$_4$	23.14 g

K$_2$HPO$_4$	135.41 g

Water	1 L

Antibiotics

Table 14: Antibiotics stock concentration

Antibiotics	1000x stock concentration	solvent
Ampicillin	100 mg/ml	Water
Kanamycin	50 mg/ml	Water
IPTG	23.83 mg/ml	Water

Buffers

6x SDS Sample buffer

SDS	1.2 g
Glycerol	4.7 ml
0.5 M Tris pH 6.8	1.2 ml
Bromophenol blue	6 mg
DTT	0.93 g
Water	2.1 ml

10x Running buffer

Tris	30.3 g
Glycine	144.0 g
SDS	10.0 g

Water	1 L

50x TAE

Tris	242 g
Acetic acid	57.1 ml
500 mM EDTA, pH 8.0	100 ml
Water	To 1 L

Miscellaneous

12% Resolving gel

Water	3.4 ml
Acrylamide/Bis 37:5:1 30%T, 2.67%C	4.0 ml
1.5 M Tris pH 8.8	2.1 ml

10% SDS	0.1 ml
TEMED	5 μl
10% Ammonium persulfate (fresh)	50 μl

(total volume = 10 ml for 2 gels)

6% Stacking gel

Water	2.7 ml
Acrylamide/Bis 37:5:1 30%T, 2.67% C	1.0 ml
0.5 M Tris pH 6.8	1.25 ml
10% SDS	50 μl
TEMED	5 μl
10% Ammonium persulfate (fresh)	25 μl

(total volume = 5 ml for 2 gels)

Coomassie Brilliant blue staining

Coomassie Brilliant Blue (R-250)	1 g
Water	400 ml
Methanol	500 ml
Acetic acid	100 ml

Coomassie Brilliant blue destaining

Water	500 ml
Methanol	400 ml
Acetic acid	100 ml

Bradford reagent

Coomassie Brilliant Blue (G-250)	100 mg

95% ethanol	50 ml
85% phosphoric acid	100 ml
Water	850 ml

1% Agarose gel

Agarose	0.5 g
TAE	50 ml
HydraGreen™ Safe DNA Dye	1 μl

Table 15: Primer list for PCR reaction

Primer number	Primer name	Primer sequence
Primer A	F-NcoI-tev-gfp-h$_6$	F 5'-ATGTCCATGGGCGAAAACCTGTATTTTCAGGGTGGTTCATCAGGTATGGTG-3'
Primer B	F-NcoI-tev-GAGAGS-gfp-h$_6$	F 5'-ATGTCCATGGGCGAAAACCTGTATTTTCAGGGTGGTGCTGGTGCTGGCTCAGGTGCTGGCTCTGGTTCATCAGGTATGGTG-3'
Primer C	F-NcoI- tev-(GAGAGS)$_2$-gfp-h$_6$	F 5'-ATGTCCATGGGCGAAAACCTGTATTTTCAGGGTGGTGCTGGTGCTGGCTCAGGTGCTGGCTCA-3'
Primer D	F-NcoI- tev-(GAGAGS)$_3$-gfp-h$_6$	F 5'-ATGTCCATGGGCGAAAACCTGTATTTTCAGGGTGGTGCTGGTGCTGGCTCAGGTGCTGGCTCAGGTGCTGGCTCAGGTGCTGGCTCAGGTTCATCAGGTATGGTG-3'
Primer E	F-(GAGAGS)$_2$-gfp-h$_6$	F 5'- GGGGGCCGGCGCTGGCTGGCTCGGGGTGCTCGGGTGCTGGCTCA-3'
Primer F	F-NcoI-tev-(GAGAGS)$_5$-gfp-h$_6$	F 5'-ATGTCCATGGGCGAAAACCTGTATTTTCAGGGTGGAGCTGGCGCCAGGTTCTGGGGCCGGCGCTGGCTCG-3'
Primer G	F-NcoI- tev-(GAGAGS)$_6$-gfp-h$_6$	F 5'-TCCATGGGCGAAAACCTGTATTTTCAGGGTGGCGCAGGCAGGCGCAGGCGGTGCTGGTGCTGGCTCAGGA-3'
Primer H	R-BamHI-gfp-h$_6$	R 5'-CGCGGATCCTTATCAGTGGTGGTGGTGGTGCTCGAGTGCG-3'
Primer I	F-NcoI-bfgf	F 5'-ATACCATGGCAGCCGGGAGCATCACCACGC-3'
Primer J	F-NcoI-h$_6$-tev-bfgf	F 5'-ATACCATGGGACACCACCACCACCACCACGGTTCCGAAAACCTGTATTTTCAG-3'
Primer K	R-HindIII-bfgf	R 5'-CGCAAGCTTTCAGCTCTTAGCAGAGACATTGGAAGAAAAGTATAGC-3'

Competent cell preparation

E.coli DH10β

E. coli DH10β was grown in 100 ml of SOB media at 30°C until OD_{600} reached 0.3. The cells were then collected by centrifugation at 6,000 xg, 4°C, for 5 minutes. After discarding the supernatant, the pellet was resuspended in 30 ml of ice-cold fresh CCMB80 buffer. After 20 minutes of incubation on ice, the cells were pelleted and resuspended in 0.04 culture volume of ice-cold CCMB80. Finally, the competent cells were aliquoted and snap freeze in liquid nitrogen and stored at -80°C.

E. coli Tuner(DE3)

E. coli Tuner(DE3) was grown in 100 ml of LB media at 37°C until OD_{600} reached 0.6. The cells were collected by centrifugation at 6,000 xg, 4°C, for 5 minutes. The pellet was then resuspended in 0.05 culture volume of ice-cold TSB buffer for 10 minutes. The competent cells were aliquoted and snap freeze in liquid nitrogen and stored at -80°C.

Determining of protein concentration by Bradford assay

BSA standard concentration in the range of 0 – 100 μg/ml (100 μl) were

added into a cuvette as 3 sets. The protein sample (100 μl) and buffer (100 μl) as

blank were added into cuvette as 3 sets. The Bradford reagent was then added (900

μl) into each cuvette of BSA standard, blank, and protein sample. The reactions were

incubated in the dark at room temperature, for 10 minutes. Absorbance at 595 nm

was then measured by spectrophotometer. Finally, the BSA standard curve was

plotted by Microsoft Excel. The protein sample concentration was determined using

A_{595} and linear regression equation obtained from linear fit of the BSA standard curve.

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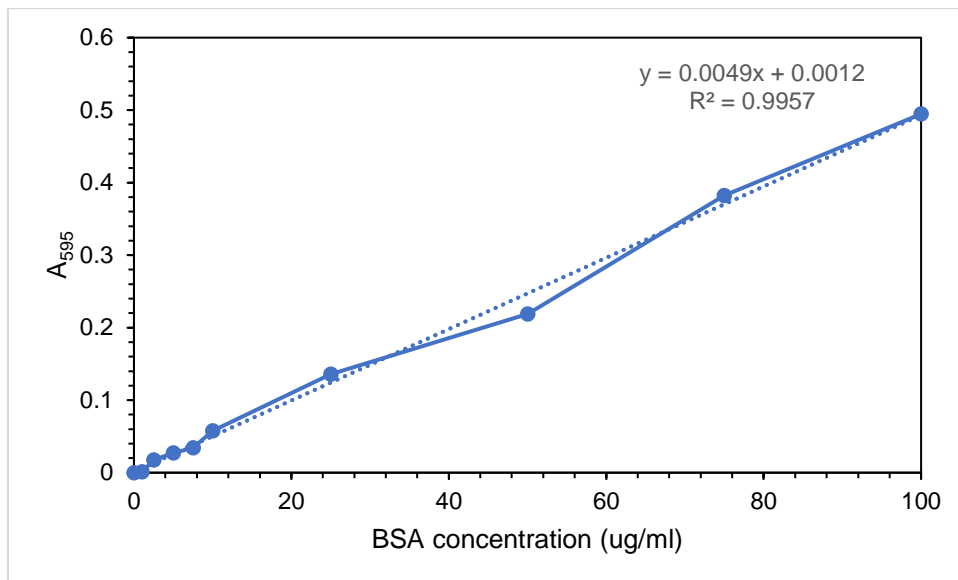CHULALONGKORN UNIVERSITY

Standard curve

Figure 24: Standard curve for (GAGAGS)$_n$-GFP

Mammalian cell culture

Mammalian cell culture media

- **Complete cell culture media**

DMEM media high glucose   250 ml

Fetal Bovine Serum   25 ml

Penicillin-Streptomycin   2.5 ml

- Non-complete cell culture media

DMEM media high glucose   250 ml

Fetal Bovine Serum   0.25 ml

Penicillin-Streptomycin   2.5 ml

Mammalian cell counting

NIH-3T3 mammalian cell lines were required knowledge of cell concentration

before seeding. The mammalian cell suspension was sampled (20 μl) was applied

onto a hematocytometer. The cells were counted in all 8 squares, then the cell

concentration was calculated using the following formula;

$$Cells\ concentration\ \left(\frac{cells}{ml}\right) = total\ counted\ cells\ x\ \frac{10^4}{number\ of\ squares}$$

MTT Assay

- **MTT solution**

MTT   100 mg

Water   10 ml

- **MTT solvent**

HCl   12.37 μl

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