DEVELOPMENT OF GELATINE-COVERED SILICA NANOPARTICLES FOR DELIVERING RECOMBINANT HUMAN SECRETORY LEUKOCYTE PROTEASE INHIBITOR (rhSLPI) FOR REDUCING MYOCARDIAL ISCHAEMIA/REPERFUSION INJURY

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Examination Committee: **Advisor:**

Ausanai Prapan ……Chairman …….

(Asst. Prof. Dr. Ausanai Prapan) (Assoc. Prof. Dr. Sarawut Kumphune)

……Member

(Assoc. Prof. Dr. Sarawut Kumphune)

Pathinan Paengnakorn ……Member

(Dr. Pathinan Paengnakorn)

A.Watcharawipha ……Member

(Dr. Anirut Watcharawipha)

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This thesis is dedicated to my family for nursing me with love and partnerships to my success.

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หัวข้อปริญญานิพนธ์	การพัฒนาอนุภาคนาโนซิลิกาห่อหุ้มด้วยเจลาตินเพื่อนำส่งโปรตีนลูกผสมตัวยับยั้งเอนไซม์ชนิดซีครีโทรีลิวโคไซต์โปรตีเอสมนุษย์ เพื่อลดการบาดเจ็บและการตายของเซลล์กล้ามเนื้อหัวใจจากภาวะขาดเลือดจำลอง
ผู้เขียน	นายฟ้าประทาน ปิกวงค์
ปริญญา	วิศวกรรมศาสตรมหาบัณฑิต (วิศวกรรมชีวการแพทย์)
อาจารย์ที่ปรึกษา	รองศาสตราจารย์ ดร.สราวุธ คำปวน

บทคัดย่อ

โรคหัวใจขาดเลือด (Ischaemic Heart Disease; IHD) เป็นสาเหตุหลักของการเสียชีวิตของประชากรทั่วโลก จากการศึกษาที่ผ่านมาแสดงให้เห็นว่า ตัวยับยั้งเอนไซม์ชนิดซีครีโทรีลิวโคไซต์โปรตีเอสมนุษย์ (Secretory Leukocyte Protease Inhibitor; SLPI) สามารถลดการตายของเซลล์กล้ามเนื้อหัวใจจากการขาดเลือดและการไหลกลับ (ischaemia and reperfusion injury ;I/R) อย่างไรก็ตาม SLPI มีครึ่งชีวิตที่สั้นเนื่องจากถูกเอนไซม์โปรตีเอส (protease)อื่นในเลือดทำลายได้ การใช้เทคโนโลยีอนุภาคนาโนจึงมีประโยชน์ในการช่วยขนส่ง SLPI ไปยังเซลล์เป้าหมายได้อย่างมีประสิทธิภาพ และจากการศึกษาที่ผ่านมามีการใช้อนุภาคนาโนหลายชนิดเพื่อห่อหุ้ม SLPI และนำไปใช้ในแบบจำลองโรคบางรูปแบบ แต่อย่างไรก็ตามยังไม่มีการศึกษาอนุภาคนาโนซิลิกาสำหรับการนำส่ง โปรตีนลูกผสม SLPI หรือ recombinant human SLPI (rhSLPI) โดยเฉพาะอย่างยิ่งในการบาดเจ็บของกล้ามเนื้อหัวใจจาก I/R ในการศึกษานี้จึงได้ทำการสังเคราะห์อนุภาคนาโนซิลิกาที่จับกับ rhSLPI และห่อหุ้มอนุภาคนาโนด้วยเจลาติน (Gelatine-covered silica nanoparticles; GSNPs) และนำไปใช้เพื่อตรวจสอบผลการลดการบาดเจ็บและการตายของเซลล์กล้ามเนื้อหัวใจจากภาวะการบาดเจ็บของการขาดเลือด/การไหลกลับจำลอง(simulated ischaemia and reperfusion injury ;sI/R) ในการศึกษานี้ได้ทำการสังเคราะห์อนุภาคนาโนของซิลิคอนไดออกไซด์ (SNPs) และบ่มด้วย rhSLPI ความเข้มข้น 0.33 มิลิกรัม/มิลลิลิตร จากนั้นนำ SNPs ที่บรรจุ rhSLPI มาเคลือบ

ด้วยเจลาติน จากนั้นทำการวัดขนาดอนุภาค ศักย์ซีตา (zeta potential) และกล้องจุลทรรศน์อิเล็กตรอน (SEM) ทำการตรวจวัดความเข้มข้นของ rhSLPI ในอนุภาคนาโนชนิด rhSLPI-GSNP และตรวจวัดการปลดปล่อย rhSLPI โดย Enzyme-linked immunosorbent assay (ELISA) ทดสอบความเป็นพิษต่อเซลล์กล้ามเนื้อหัวใจของหนูแรท (H9c2 cell) และทดสอบผลในการลดการบาดเจ็บและการตายของเซลล์กล้ามเนื้อหัวใจในภาวะการขาดเลือดและการไหลกลับจำลอง ผลการศึกษาพบว่า SNPs GSNPs และ rhSLPI-GSNP มีขนาด 273 300 และ 301 นาโนเมตร ตามลำดับ และมีค่าศักย์ซีตาเท่ากับ -57.21 -22.40 และ -24.50 mV ตามลำดับ rhSLPI-GSNPs น้ำหนัก 1 มิลลิกรัม สามารถตรวจพบ rhSLPI 235 นาโนกรัม ผลการทดสอบความเป็นพิษของอนุภาคนาโนต่อเซลล์กล้ามเนื้อหัวใจพบว่า อนุภาคนาโนที่พัฒนาขึ้นไม่มีความเป็นพิษต่อเซลล์ และ rhSLPI-GSNPs ที่พัฒนาขึ้นสามารถลดการบาดเจ็บและการตายของเซลล์กล้ามเนื้อหัวใจจาก sI/R โดยสรุป การศึกษานี้เป็นครั้งแรกที่สร้างอนุภาคนาโนเจลาตินห่อหุ้มซิลิกอนไอออกไซด์ที่บรรจุ rhSLPI (rhSLPI-GSNPs) และให้ผลการป้องกันหัวใจโดยการต่อต้านภาวะการบาดเจ็บของการขาดเลือด/การไหลกลับจำลองในหลอดทดลองในเซลล์หัวใจ

Thesis Title	Development of Gelatine-covered Silica Nanoparticles for Delivering Recombinant Human Secretory Leukocyte Protease Inhibitor (rhSLPI) for Reducing Myocardial Ischaemia/Reperfusion Injury
Author	Mr. Faprathan Pikwong
Degree	Master of Engineering (Biomedical Engineering)
Advisor	Associate Professor Dr. Sarawut Kumphune

ABSTRACT

Ischaemic Heart Disease (IHD) is the main global cause of death. Previous studies indicated that recombinant human secretory leukocyte protease inhibitor (rhSLPI) exhibits a cardioprotective effect against myocardial ischaemia/reperfusion (I/R) injury. However, SLPI has a short half-life *in vivo* due to digestion by protease enzymes in circulation. Application of nanoparticle encapsulation could be benefit for SLPI delivery. Several types of nanoparticles have been developed to encapsulate SLPI and applied in some disease models. However, silica nanoparticles for rhSLPI delivery, particularly on myocardial I/R injury, has never been studied. In this study, rhSLPI was encapsulated into gelatine-covered silica nanoparticles (GSNPs), which will be further used to determine an *in vitro* cardioprotective effect against simulated ischaemia/reperfusion injury (sI/R) in rat cardiac myoblast cell line (H9c2). Silica dioxide nanoparticles (SNPs) were fabricated followed by incubation with 0.33 mg/mL of rhSLPI. Finally, SNPs containing rhSLPI were coated with gelatine (GSNPs). The GSNPs and rhSLPI-GSNPs were characterized by particle size, zeta potential, and morphology scanning electron microscope (SEM). The concentration of rhSLPI in rhSLPI-GSNPs and drug release were determined by Enzyme-linked immunosorbent assay (ELISA). Then, cytotoxicity was determined by incubation of GSNPs or rhSLPI-GSNPs with rat cardiac myoblast cell line (H9c2). The results showed that the particle size of SNPs, GSNPs, and rhSLPI-GSNPs

were 273, 300, and 301 nm, respectively, with zeta potential of -57.21, -22.40, and -24.50 mV, respectively. One milligram of rhSLPI-GSNPs contained 235 ng of rhSLPI. The cytotoxicity showed no significant difference between cardiac cells. The rhSLPI-GSNPs provided cardioprotective effect to reduce cardiac cell injury and death against sI/R. In conclusion, this is the first study of rhSLPI encapsulated gelatine-covered nanoparticles (rhSLPI-GSNPs) synthesis. rhSLPI -GSNPs provides cardioprotective effect to against an *in vitro* sI/R in cardiac cells.

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LIST OF ABBREVIATIONS

CVD	Cardiovascular diseases
WHO	World Health Organization
CAD	Coronary artery disease
PAD	Peripheral artery disease
IHD	Ischaemic heart disease
MI	Myocardial infarction
I/R	Ischaemia/reperfusion
sI/R	Simulated ischaemia/reperfusion
ATP	Adenosine triphosphate
mPTP	Mitochondrial permeability transition pore
ER	Endoplasmic reticulum
SR	Sarcoplasmic reticulum
ROS	Reactive oxygen species
PSGL-1	P-selectin glycoprotein ligand-1
STEMI	ST-elevation myocardial infarction
NSTEMI	Non-ST elevation myocardial infarction
IL-1	Interleukin 1
IL-6	Interleukin 6
CRP	C-reactive protein
COX-2	Cyclooxygenase-2
MMP	Matrix metalloproteinases
Apaf-1	Apoptosis protease activation factor 1
MPO	Myeloperoxidase
PMNs	Polymorphonuclear cells
WAP	Whey acidic protein
NE	Neutrophil elastase
TIMP	Tissue inhibitors of metalloproteinases

TLRs	Thymic stromal lymphopoietin
LPS	Lipopolysaccharides
TNF-α	Tumour necrosis factor alpha
TSLP	Thymic stromal lymphopoietin
ECM	Extracellular matrix
NF-κB	Nuclear factor-κB
COPD	Chronic obstructive pulmonary disease
CF	Cystic fibrosis
EGFR	Epidermal growth factor receptor
TGF-β	Transforming growth factor beta
CI	Cold ischaemia
ECs	Endothelial cells
HAS	Human serum albumin
PBS	Phosphate-buffered saline
MSN	Mesoporous silica nanoparticles
DOX	Doxorubicin
SLPI	Secretory Leukocyte Peptidase Inhibitor
rhSLPI	Recombinant Human Secretory Leukocyte Peptidase Inhibitor
SNPs	Silica nanoparticles
GSNPs	Gelatine covered silica nanoparticles
rhSLPI-GSNP	Recombinant human Secretory Leukocyte Peptidase Inhibitor gelatine covered silica nanoparticles
SEM	Scanning electron microscope
FE-SEM	Field emission scanning electron microscopy
LDH	Lactate Dehydrogenase
ELISA	Enzyme-linked immunosorbent assay
PDI	Polydispersity Index
TEOS	Tetraethyl orthosilicate
DMEM	Dulbecco's Modified Eagle Medium
FBS	Foetal bovine serum

DI water	Deionized Water
EE%	Percentage of Encapsulation efficiency
HRP	Horseradish peroxidase
DMSO	Dimethyl sulfoxide
DLVO	Derjaguin–Landau–Verwey–Overbeek
pSiNP	Porous silica nanoparticles
GMSNs	Gelatine covered mesoporous silica nanoparticles

CHAPTER 1

Introduction

1.1. Background and problems

Ischaemic Heart Disease (IHD) is predicted to be the major cause of death in 2030 [1]. IHD is the main cause of mortality, which is responsible for approximately 20% of the world's total deaths and more than 9 million deaths in 2019 [2]. In lower-middle-income countries, IHD is top 10 causes of death [2]. Similarly, IHD is the leading cause of death among Thailand's top 10 major causes of mortality, and it is increasing every year [3]. IHD is caused by myocardial infarction (MI), a serious condition caused by necrosis in a section of the heart muscle due to insufficient coronary blood flow [4]. Myocardial infarction primarily causes by myocardial ischaemia, which is referred to as a lack of blood supply to the heart tissue. During ischaemia, several biochemical and physiological changes occur and could cause an injury, which is the so-called ischaemic injury. The best treatment is known as "reperfusion therapy", which also aggravates cellular damage [4]. An injury that occurs during reperfusion is known as "Reperfusion injury." Taken together, the injury in both ischaemia and reperfusion are the so-called "Ischaemia/Reperfusion (I/R) injury" that is the most challenging research question for scientists who are interested to cure heart attack. While occurring myocardial ischaemia and reperfusion, numerous biochemical processes take place, such as the release of proteases, polymorphonuclear cell infiltration, and post-ischaemic inflammation. These result in tissue damage, cell necrosis, and subsequently impaired function [5, 6]. Protease enzymes have a lengthy half-life in tissue and cause extensive damage [7]. Moreover, An excessive activity of protease enzymes such as chymase, calpains, cathepsins, matrix

metalloproteinases, and caspases contributes to the process of cell death, injury, and cardiac remodeling, which is a main cause of heart failure [8].

Inhibition of protease might be considered a powerful strategy for the prevention of ischaemia/reperfusion (I/R) induced tissue injury, the progression of cardiac hypertrophy, and heart failure. Thus, using protease inhibitors to reduce protease activity could have therapeutic potential. Despite the progression of numerous small molecule protease inhibitors, the "off-target effect" of these potential therapeutic candidates appears to be of grave concern [9]. Alternatively, the endogenous anti-protease peptide, which are normally expressed and functions in the human body, could provide more reliable results and be safe.

Typical endogenous anti-serine protease protein such as a secretory leukocyte protease inhibitor (SLPI) is secreted from body fluids, including saliva, tears, bronchial secretions, seminal fluids, and intestinal mucus [10]. SLPI is a selective inhibitor for serine protease such as elastase and cathepsin G from neutrophils, and chymase and tryptase from mast cells [11]. In addition to controlling the processing of inflammation, SLPI also protects the host from diffuse tissue damage caused by proteolytic enzymes generated while occurring inflammation and suppresses inflammatory responses [11]. Our previous studies demonstrated that recombinant human SLPI (rhSLPI) that is overexpressed the rhSLPI gene or treatment with recombinant protein of human SLPI provided cytoprotective effect against an *in vivo, ex vivo,* and *in vitro* I/R injury [12-15]. However, previous studies showed that SLPI was discovered to have a rapid clearance rate in both human plasma and urine. SLPI is normally eliminated through glomerular filtration, then reabsorption and degradation in tubular cells, and within 2-6 h interval, the estimated half-life of SLPI in plasma is 120 min [16]. Besides, SLPI is inactivated by an enzyme that is secreted from the respiratory tract such as cathepsin [17]. Thus, the transportation of SLPI to the target organ is a new challenge. Therefore, any strategies to improve the stability and extend the half-life of SLPI in the system could provide greater therapeutic benefit.

Several advantages of nanoparticles for peptide drug carriers have been listed, for example reducing the enzymatic digestion, aggregation of peptide drugs, and increasing the transmembrane absorption. Nowadays, several types of nanoparticles have been studied such as gelatine and silica. Gelatine-covered silica nanoparticles (GSNPs) are extremely effective at delivering and controlling the release of medications, proteins, and peptides. The delivery approach based on GSNPs is biocompatible and biodegradable in the body, with no hazardous breakdown products [18]. Gelatine is a polyampholyte that contains cationic, anionic, and hydrophilic groups as well as a hydrophilic group [19]. Mechanical features of gelatine NPs, including swelling behaviour and thermal properties, are known to be influenced by the degree of cross-linking between cationic and anionic groups [19].

Growing evidences of gelatine-covered silica nanoparticles suggest the benefit as a drug delivery carrier. However, the efficiency of gelatine-covered silica nanoparticles for rhSLPI delivery, as a peptide drug, especially for cardiac cells subjected to I/R injury has never been investigated.

1.2. Objective

1.2.1. To prepare gelatine-covered silica nanoparticles (GSNPs) for rhSLPI delivery.

1.2.2. To determine the cardioprotective effects of rhSLPI-loaded GSNPs (rhSLPI-GSNPs) against an *in vitro* simulated ischaemia/reperfusion injury in H9c2 cell.

1.3. Hypothesis

1.3.1. GSNPs are able to encapsulate rhSLPI.

1.3.2. SLPI- GSNPs are able to reduce H9c2 cell injury and death under *in vitro* simulated ischaemia/reperfusion injury.

1.4. Research scope

The study focuses on preparation of rhSLPI-loaded nanoparticles by rhSLPI into gelatine-covered silica nanoparticles. Nanoparticles were examined for physical characteristics, morphology, drug release, and cell cytotoxicity. Myocardial ischaemia/reperfusion injury was performed as an *in vitro* simulated I/R injury in a rat cardiac myoblast cell line (H9c2).

CHAPTER 2

Literature Review

2.1. Myocardial ischaemia

Cardiovascular diseases (CVD) are the main cause of mortality globally [20]. The World Health Organization (WHO) has suggested that 18 million people was killed by CVD in 2019, accounting for 32% of deaths. Heart attacks caused 85 percent of deaths [21]. Most low-income countries and middle-income countries, including Thailand, are experiencing an epidemiologic shift, with a sharp increase in noncommunicable diseases [3]. According to the WHO, CVD will kill 24.2 million people every year by 2030 [22]. In Thailand, CVD has become a significant cause of death and is prospective to continue to be a significant cause of morbidity and mortality in the future.

Obstructive blood vessel is a condition that involve cardiovascular disease (CVD) such as ischaemic heart disease (IHD), which is a condition caused by inadequate blood and oxygen to a part of the myocardium. IHD is concerned because it is the most cause of death in CVD for an estimated 13 million people in 2010 [23] due to cardiac tissue is damaged and died, resulting in an acute myocardial infarction and heart attack.

2.2. Definition of myocardial ischaemia

The term “ischaemia” mean “withholding of blood” that was coined by Rudolph Virchow in 1858 to describe reduced perfusion of an organ or tissue with its consequences [24]. Hearse, Eric Feigl, and John Ross defined that myocardial ischaemia is induced by blocked blood flow that has functional and/or metabolic consequences for the affected myocardium [25]. The myocardial lacks oxygen due to coronary artery blockage, which leads to cellular necrosis in the heart tissue. All of these are called myocardial ischaemia.

2.3. Mechanism of ischaemic injury

When ischaemia is occurring anaerobic metabolism takes over, resulting in pH decrease in a cell. Excessed hydrogen ions excrete excess to cytosol to compensates an accumulation of hydrogen ions by the Na^+/H^+ exchanger, resulting a large influx of sodium ions the cell. Ischaemia reduces cellular ATP by inhibited the Na^+/K^+ ATPase, reduce a Ca^{2+} efflux, and restricted calcium reuptake of the endoplasmic reticulum (ER), resulting a calcium overload in the cell. Moreover, there are occurred to parallel with the closing of the mitochondrial permeability transition pore (mPTP), which induces mitochondrial membrane damage [4]. These cellular changes in the heart are accompanied by the activation of intracellular proteases such as calpains, which damage myofibrils and cause hyper contracture and cell death [15]. The extent of these changes, and the degree of tissue injury, follows the size of the blood supply reduction and the length of the ischaemic period. Other biochemical processes that are unrelated to ischaemic injury take place during ischaemia, but when oxygen come back, elements are again delivered to the tissues, they set off a series of processes known as reperfusion injury, which increases tissue damage [15, 26]. (Figure 1)

2.4. Mechanism of reperfusion injury

In reperfusion, the respiratory activity, mitochondrial potential, and synthesis of ATP are restored, and intracellular pH is rapidly restored to normal levels, causing calpain, a Ca^{2+}-dependent protease that destroys the cytoskeleton and sarcolemma, to be activated [27]. In the presence of increasing Ca^{2+}, the increased availability of ATP promotes Ca^{2+} uptake of sarcoplasmic reticulum (SR), exceeding the threshold of channels, which release Ca^{2+} into the cytoplasm. This process is repeated cyclically, resulting Ca^{2+} oscillations, this cause uncontrolled myofibrillar hypercontraction and promote the opening of mPTP [28]. When mPTP is activated, the mitochondrial matrix swells, this causes the outer membrane to rupture and the contents of the mitochondrial intermembrane space, such as cytochrome c, the leakage to the cytosol. Increased Ca^{2+} oscillations also boost xanthine oxidase activity, promoting the production of reactive oxygen species (ROS), which

exacerbates damage of membrane by promoting mPTP opening. In the ischaemia zone, cells such as damaged myocytes, endothelial cells, and neutrophils produce the majority of ROS. By releasing inflammatory mediators like elastase upon entering the ischaemia zone, neutrophils further exacerbate cellular damage, leading to microvascular blockage, local inflammation, and ultimately systemic inflammation [29]. (Figure 1)

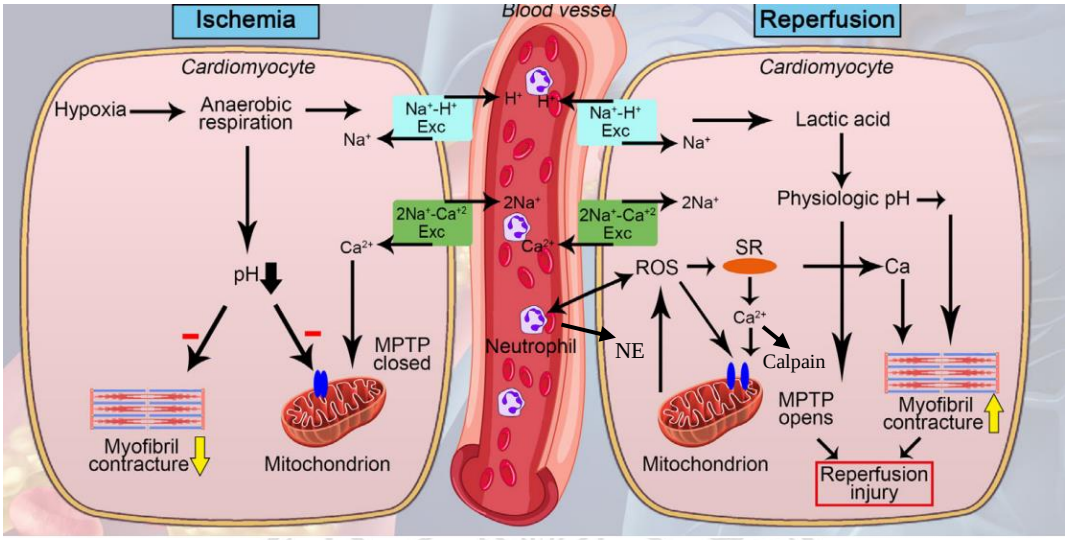


Figure 1 Pathogenesis of myocardial ischaemia/reperfusion injury. When ischaemia is occurring, cells use anaerobic metabolism. As the result, pH is decreased. The Na+/H+ exchanger excretes excess hydrogen ions to buffer this accumulation of hydrogen ions, resulting in a large influx of sodium ions, which exchange with calcium ions. causing calcium overload in the cell. mPTP is closed by low pH and accumulation of calcium. Reperfusion is overloaded calcium, which helps calpain activity. mPTP is opened by retained pH and overloaded calcium. mPTP releases caspases that make apoptosis. Blood flow will induce ROS production, which is the cause of injury [4]. (Modified from Gunata M. Cell Biochemistry and Function. 2021)

2.5. Therapeutic approaches for I/R injury

Numerous promising cardioprotective medications that target platelets, inflammation, oxidative stress, calcium overload, and pH correction in the I/R. Targeted pharmacotherapy is used in treatment of injury [30]. A calcium overload that is one targeting to be reduced by a pharmacological antagonist, which target on sarcolemmal Ca^{2+} ion channel [31]. Calcium antagonists including ethylene glycol bis (2-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), nifedipine, diltiazem, methadone, verapamil, and morphine provide reducing of myocardial injury *in vivo* models [31, 32]. Pharmacologic inhibitors of protease, which is leukocyte treatments that target neutrophils during reperfusion have been effective in reducing the infarct size. P-selectin inhibitors is pharmacologic inhibitors of protease, it can bind to P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1), which present on neutrophils. P-selectin inhibitors decrease an pro-inflammatory cytokine expression and I/R by preventing leucocytes from adhering to endothelial cells, they is reduced their penetration into the vessel membrane [33]. Treatment with P-selectin inhibitors (inclacumab) was linked to significantly lower plasma troponin I levels in patients with NSTEMI undergoing percutaneous coronary intervention (PCI) [34]. Complement inhibitors, which is pexelizumab, a C5 complement protein inhibitor is not have an effect on infarct size but in STEMI patients receiving fibrinolysis, however, it significantly reduced mortality [35]. Colchicine is anti-inflammatory drug, which have been used for gout. Colchicine, which anti-inflammatory properties inhibits leukocytes, and endothelial cells, it inhibits the production of inflammatory mediators such as proteolytic enzymes. Colchicine use showed reducing of IL-6 and high sensitive CRP in patients [36]. IL-6 receptor antagonist (tocilizumab), which is one of pharmacological antagonist can decrease the level of CRP and Troponin T (TnT) levels in a mice experiment of myocardial infarction [37] and IL-1 receptor antagonist (anakinra) can reduce significantly lower level of CRP in patients [38]. Myocardial adenosine levels have been linked to improvements in tissue remodeling, decreased production of inflammatory markers, decreased infarct size, oedema, and increased coronary blood flow [39]. Adenosine acts by activating the adenosine A2A

receptor, which in turn upregulates the activity of the cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS) [40].

2.6. Protease enzyme in I/R injury

Protease enzyme is a part of myocardial I/R injury, which was promoted by oxygen insufficiency. Main factor contributing to myocardial cell damage after I/R is protease enzymes, which was produced by injured cells and leukocytes [41]. A main mechanism of the heart failure progression is the myocardial infarction, which is caused by increasing in the proteolytic activity of protease such as chymase, matrix metalloproteinases (MMP), calpains, cathepsins, and caspases (Figure 2) [8]. The calpain is closely associated with myocardial infarction because of its specially degradative and pro-apoptotic properties, which are a major cause of the heart injury brought on by oxidative stress and Ca^{2+} overload [8]. In cardiac cells, it has shown that calpain could reduce the activity of Na-K-ATPase resulting in apoptosis [42]. The progression and development of various cardiac pathologies have all been linked to cathepsins. The risk of cell damage as well as the amount of direct myocardial damage caused by cathepsins, which are increased [43]. Caspases is one of key in cell apoptosis. The caspases are crucial for apoptosis regulation, which is activated by the mitochondrial pathway. The most crucial apoptotic protease is caspase 3 [44], which was produced by procaspase-9 was activated into caspase-9 by the interaction of apoptosis protease activation factor 1 (Apaf-1), and cytochrome c, as a result of the upstream activation of caspases (Caspase-3, 6, and 7) to increase apoptosis [45]. MMP, which is widely produced and broadly distributed in almost heart cells and is an integral sarcomeric protein that participates in the proteolysis of sarcomeric and cytoskeletal proteins that are weakened, and which can be activated by oxidative stress [46]. The critical phase of neutrophil degranulation during I/R was essential to their regulatory role [47]. Neutrophil degranulation is releasing granules containing cathepsin G, serine proteases, neutrophil elastase (NE), and myeloperoxidase (MPO) and inducing the injury of myocardial infarction [47]. Neutrophil secrets NE, which lead to cardiac damage by digestion of elastin, collagen, and fibrinogen [48]. pro-MMP-9, which is one of the MMP family members is activated by NE, it has been studied the most in relation

to myocardial infarction, illustrating the interaction of molecules derived from PMNs [49, 50]. One of the primary functional proteins in neutrophils, it is myeloperoxidase (MPO), makes up about 5% of their dry weight [51]. Degranulation or attachment to the neutrophil extracellular trap are about 30% of MPO that is contained in the azurophilic granules can be released extracellularly [52]. MPO is express in monocytes less concentrations than in neutrophils. [52, 53].

Obviously, Protease enzymes provide terrible effects in I/R injury. Thus, inhibitions of proteases could provide cardioprotection to myocardial injury. In 2008, J. Carter, one of protease inhibitors that use to reduce myocardial injury at I/R is Aprotinin, which is a serine protease inhibitor, which reduce necrotic area of MI [54]. Another one of serine protease inhibitor is secretory leukocyte protease inhibitor (SLPI), which can inhibits NE, trypsin, cathepsin G, and chymotrypsin [55], it provide cardioprotective effect on I/R injury and is crucial for the restoration of organ function quickly following I/R and heart transplantation, and that it inhibits the post-ischaemic inflammatory response [56]. However, the risk of Aprotinin in cardiac surgery has reported complications in Cardiovascular and myocardial infarction (Figure 3), low dose and high dose of Aprotinin showed complication in cardiovascular [57] (Figure 4). Other protease inhibitor that use to reduce myocardial injury is chymase inhibitor, which is candidate in I/R. Chymase inhibitor could provide cardioprotective effect by inhibiting activation of matrix metalloproteinase-9 (MMP-9) [58], and chymase entry into the cardiomyocyte [59]. However, Arooj M, at el. has reported that chymase inhibitor has off-target to several systems, it's small molecule can be linked to several pathways such as complement system, coagulation, and fibrinolytic system as well as off-target of diseases such as cancer, haemorrhage, thrombosis and central nervous system diseases [9] (Figure 5). Therefore, new protease inhibitor that is body friendly protease inhibitor might be benefit for reducing immunogenicity and can reduce damage from I/R injury, it could be requested.

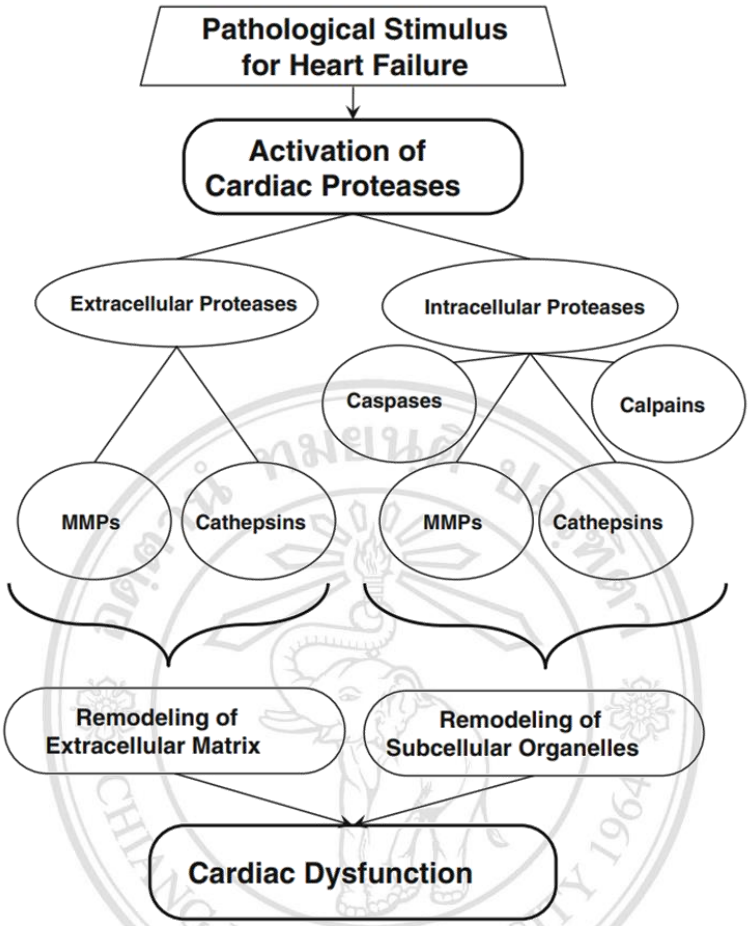


Figure 2 Protease enzymes lead to cardiac dysfunction [8].

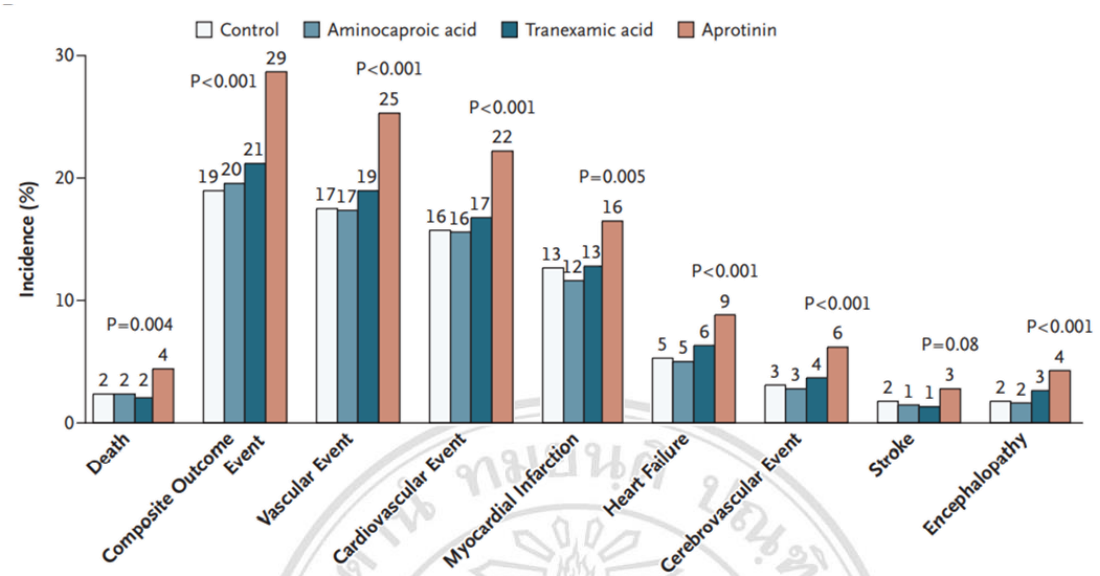


Figure 3 Complications of Aprotinin [57].

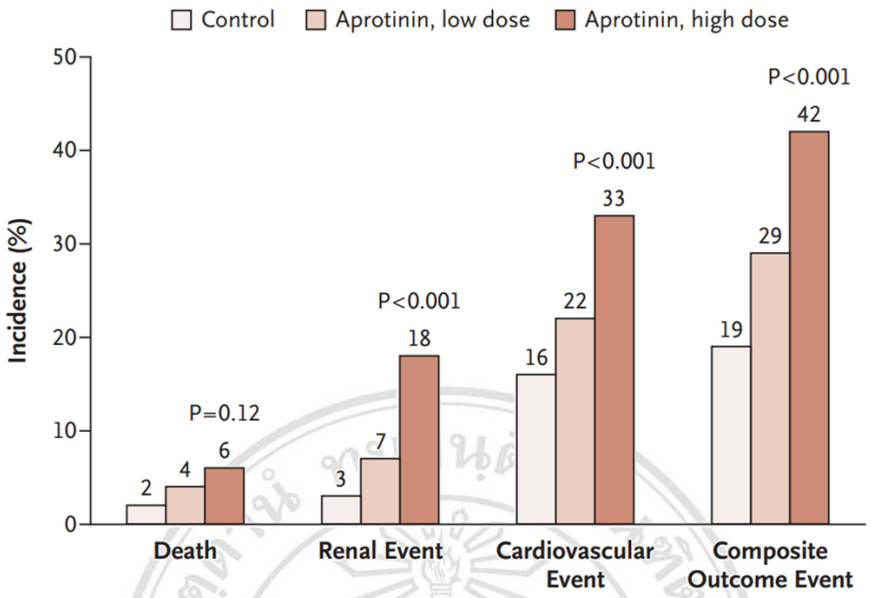


Figure 4 Complications of low dose and high dose of Aprotinin [57].

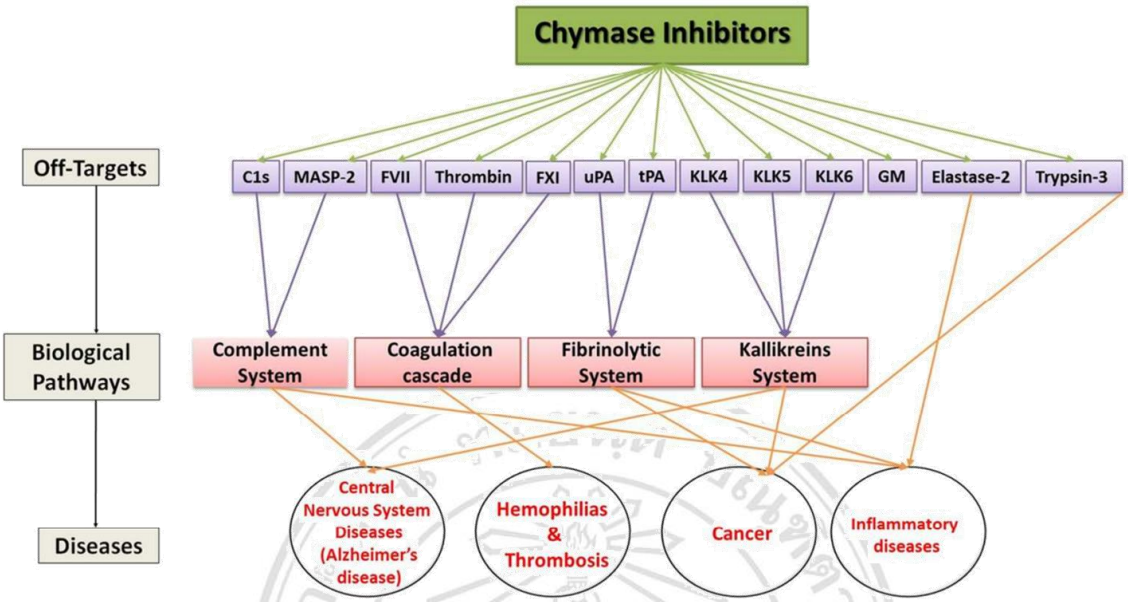


Figure 5 Off-targets of chymase inhibitor [9].

2.7. Secretory leukocyte protease inhibitor (SLPI)

The secretory leukocyte protease inhibitor (SLPI) is found in mucosal secretions and is a significant contribution to the antiprotease screen [60]. The major characteristic of SLPI is anti-protease ability that inhibits serine proteases, such as cathepsin G, and neutrophil elastase, tryptase, and chymase. In addition to being produced by host-defence effector cells, this inhibitory protein is expressed and secreted by epithelial cells. The protein SLPI is 107 amino acids long and has homologous whey acidic protein (WAP) domains. There are cysteine residues in this domain, which together produce four intramolecular disulfide bonds. WAP can separate 2 parts, including WAP I domain (N-terminal) is antimicrobial activity, and WAP II domain (C-terminal) is inhibitory activity against proteases, which protease-inhibiting region of SLPI was located residues 67 to74 amino acids [11]. (Figure 6)

Typically, SLPI is released by epithelial cells bordering mucous membranes in the respiratory, digestive, and reproductive tracts. It can be found in whole saliva [61], cervical mucus [62], seminal fluid [63], synovial fluid [64], breast milk [65], tears, and cerebral spinal fluid [66], among other mucosal fluids, as well as secreted by neutrophils and macrophages.

SLPI is an anti-protease, which is secreted by cells at the site of inflammation to maintain tissue homeostasis. The accumulation of polymorphonuclear cells (PMNs), the particularly neutrophil influx, causes the NE releases, which causes the production of many proinflammatory factors such as interleukin 8 (IL-8) [67]. These include metalloproteinase activation and inactivation of their inhibitors, tissue inhibitors of metalloproteinases, which increases the proteolytic potential. Eventually, ECM degradation increases. One of the mechanisms that control the extent of NE action is epithelial cell secretion of SLPI [67].

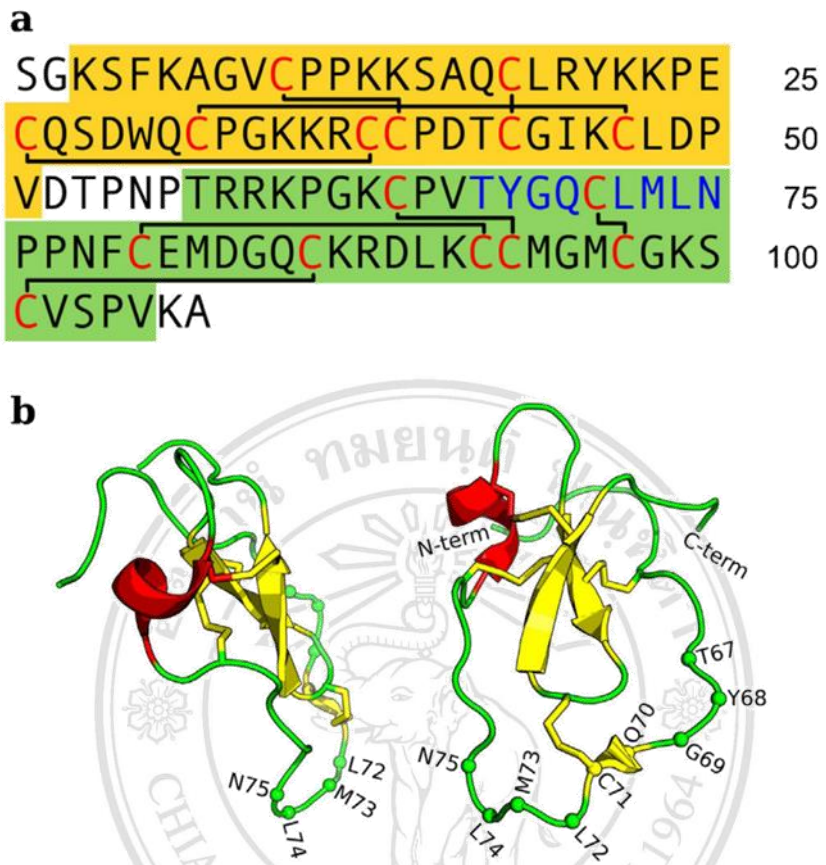


Figure 6 Amino acid sequence of SLPI. (a) Amino acid sequences of two WAP domains. Cysteine residues represented in red produce intradomain disulfide bridges (black lines). (b) The WAP II domain match the protein region involved in protease interactions that is illustrated in two orthogonal perspectives in yellow and red, with three b-strands and a short helix [11].

2.8. Regulation of SLPI expression

Many proinflammatory of microorganism stimulation activate cell by capturing to pattern recognition receptors like Toll-like receptors (TLRs), which stimulate increasing SLPI expression [68]. One of these stimuli are bacteria, which contain elements of bacterial cell walls such as Lipopolysaccharides (LPS) and lipoteichoic acid [69]. TNF-α, IL1-β, and thymic stromal lymphopoietin (TSLP)-provided cytokine signals regulate the expression of SLPI [70]. The primary enzymatic target of SLPI, such as NE, which increases the production of SLPI during granulopoiesis [71]. One of intracellular signaling cascades involved in controlling SLPI production is the cAMP-dependent pathway because SLPI is upregulated in response to elevated intracellular cAMP levels [71]. LEF-1 and C/EBP-α, transcription factors, may act as downstream regulators of SLPI [72]. LEF-1 has been demonstrated to control SLPI mRNA levels in the myeloid cell line (NB4), most likely through LEF-1-mediated activation and C/EBP- α binding to the SLPI promoter [11, 69, 72].

2.9. Function of SLPI

2.9.1. Enzymatic activity of SLPI

An inhibition of NE is the purpose of a part of SLPI. The fact that SLPI is in charge of approximately 90% of the NE inhibition of upper respiratory tract secretions indicates that it is crucial for controlling local NE activity. Typically, NE release from activated neutrophils, NE either processes growth factors and ECM components or digests proteins within specialized neutrophil granules. As a result, SLPI protects proteins from NE digestion as a regulating inhibitor of NE. This protection has significant implications for the initiation and proper termination of inflammatory responses [11, 73].

2.9.2. NF-κB inhibition of SLPI

SLPI play role in controlling inflammatory responses, which has clearly been described extend beyond the inhibition of serine proteases. The transcription factor of NF-κB is inactivated by SLPI, which reduces the monocyte and macrophage response to LPS [11]. The fundamental mechanism of this inhibition is that it prevents SLPI from degrading NF-κB inhibitory components like IκBα and IκBβ [11]. SLPI competes with the NF-κB component p65 for NF-κB sites in the promoters of proinflammatory genes such as TNF-α and CXCL8. NF-κB regulates immunological and proliferative/survival responses, and SLPI can maintain homeostasis at bodily barriers or inflammatory processes [74].

2.9.3. Antimicrobial activity of SLPI

The N-terminal (WAP I) domain of SLPI has been attributed to the majority of SLPI's antimicrobial activity, which can determinate both of gram-positive bacteria and gram-negative bacteria, as well as fungus. SLPI kill microorganism similar to other cationic peptides [11]. The C-terminal domain of SLPI have very poor bactericidal activity while having substantially lower antifungal characteristics than the N-terminal domain. SLPI can disrupt microbial membranes by interacting with anionic bacterial lipids because to the WAP's strong positive charge. Normally, the N-terminal domain of SLPI is cations charge (+7) and has significantly stronger microbicidal activity than its C-terminal domain, which is less positively charged (+5) [11, 75]. SLPI can block HIV-1 infection by capturing with annexin II which is a cofactor of HIV-1 infection and thereby disrupts the interaction between HIV-1 and macrophages. Moreover, SLPI can prevent CD4+, the primary HIV-1 receptor on CD4+ and macrophages, from interacting with the membrane proteins phospholipid scramblase 1 and 4 of host [76].

2.9.4. SLPI and respiratory tract

SLPI can express by bronchial epithelial cells in the lungs. SLPI can be responsible for the antiprotease activity such as anti-elastase in the upper respiratory tract [77]. SLPI express in the airway at 30-fold higher levels than superficial epithelium [78]. Chronic obstructive pulmonary disease (COPD) and Cystic fibrosis (CF) are influenced by an imbalance of proteases and protease inhibitors [79]. Antiprotease of SLPI reduces lung destruction of neutrophil by inactivating NE and reducing production of IL-8 in lung epithelial cells [80]. In Bronchoalveolar lavage (BAL, there are no differences in SLPI concentrations between CF patients and healthy individuals [81].

2.9.5. SLPI and skin

SLPI play role in wound of skin, which has been demonstrated in the wound healing. The wound promotes SLPI releasing in skin cells via a epidermal growth factor receptor (EGFR) [82]. In the damaged skin of SLPI mutant mice, wound healing is delayed due to increased elastase activity, neutrophil and monocyte accumulation, and TGF-β activation [83]. TGF-β neutralization is irreversible to the delayed wound healing in SLPI knockout mice as well as exogenous SLPI, implying that SLPI enhances wound healing through mechanisms other than TGF-β suppression [83]. SLPI aids wound healing by inhibiting NE from converting progranulin to granulin peptides of neutrophils [73]. TNF-α activation of neutrophils is blocked by progranulin, an epithelial growth factor. Granulin peptides, on the other hand, cause IL-8 production and so attract neutrophils. In SLPI knockout mice, progranulin restores wound healing [73]. SLPI prevents excessive collagen contraction by human fibroblasts originating from hypertrophic scar tissue, which may help to avoid scarring following wound healing [81].

2.9.6. SLPI and gastrointestinal tract

SLPI is released from the intestine epithelial cell, in both the Paneth cells and goblet cells disrupted epithelial cell. Microbial interaction induces SLPI production in epithelial cells of intestine. SLPI is Secreted in epithelial cells, which is activated by TNF-α, IL-1β, and the protein kinase C pathway [84]. Even though SLPI in saliva is rapidly broken down in the stomach and duodenum, it is likely that SLPI in an intestinal fluid is produced locally. Due to the SLPI low concentration in luminal fluid, SLPI is secreted from epithelial cells, it has its effects locally at the surface of epithelial cells [73, 84]. SLPI inhibits inflammation during intestinal immune reactions. SLPI prevents leukocyte infiltration at heavily covered mucosal surfaces by inhibiting NF-κB signaling in intestinal epithelial cells [85].

2.9.7. SLPI and Cardiovascular diseases

In 2008, Schneeberger et al. firstly demonstrated that SLPI could benefit for organ functions early after I/R and cardiac transplantation, it has an inhibitory effect on the post-ischaemic inflammatory response [56]. The heart exposure to impair myocardial contraction, which was related to increased protease enzyme production in the heart. In addition, there was a considerable rise in inflammation, myocyte vacuolization, and cellular necrosis in the hearts. SLPI can reduce myocardial cell death and injury *in vitro* simulated I/R (sI/R) condition [12]. Previous studies showed that SLPI can protect cardiac fibroblasts subjected to I/R conditions *in vitro* study [14]. In I/R injuries and responses, signal transduction is critical. In the aetiology of I/R damage, numerous protein kinases and cellular apoptotic regulatory proteins have been implicated. In I/R damage, the p38 MAPK, Bax, Bcl-2, and caspase cascades have all been identified as mediators of cellular apoptosis. The overexpression of SLPI in cardiomyocytes or treatment of SLPI in isolated hearts reduce cell death, and injury, as well as reduced infarct size [14].

Vascular endothelial cells that overexpress and secrete SLPI can shield endothelial cells and cardiomyocytes from I/R damage. SLPI increases Akt phosphorylation and activates p38 MAPK while decreasing intracellular ROS production and p38 MAPK phosphorylation. [86]. Endothelial cell (ECs) can be made to secrete rhSLPI, which protects the heart and blood vessels from I/R damage. By reducing expression of ROS in the cell, the Bax/Bcl-2 ratio, caspase-3, and caspase-8, preconditioning of endothelial-derived rhSLPI by activation of p38 MAPK and Akt has the potential to lessen cardiac cell and endothelial cell death. Furthermore, rhSLPI could be used as a novel or alternative additive component in the organ preservative solution for transplantation to increase the quality of isolated graft tissue, particularly vascular or heart tissue [86]. In 2021, Mongkolpathumrat P, et al. administered SLPI after inducing myocardial ischaemia *in vivo* rat model. The study demonstrated that *in vivo* treatment of SLPI could reduce infarct size, and inflammatory cytokines, and oxidatively modified proteins, as well as apoptosis regulatory signaling [87]. However, the cardioprotective effect of SLPI is still questionable that the benefit is dependent on its anti-protease activity. The anti-protease activity deficient mutant was generated and could still provide cardioprotection effect in rat model of myocardial I/R injury [88]. Furthermore, cardiac specific overexpression of SLPI by adeno-associated virus (AAV) serotype 9 as gene delivery was also implemented in myocardial I/R model in rats. The AAV9-SLPI could significantly reduce inflammatory cytokines, infarct size, and production of ROS, as well as cardiac biomarker such as cardiac troponin I, CK-MB, and myoglobin and improve cardiac function [89]. (Figure 7)

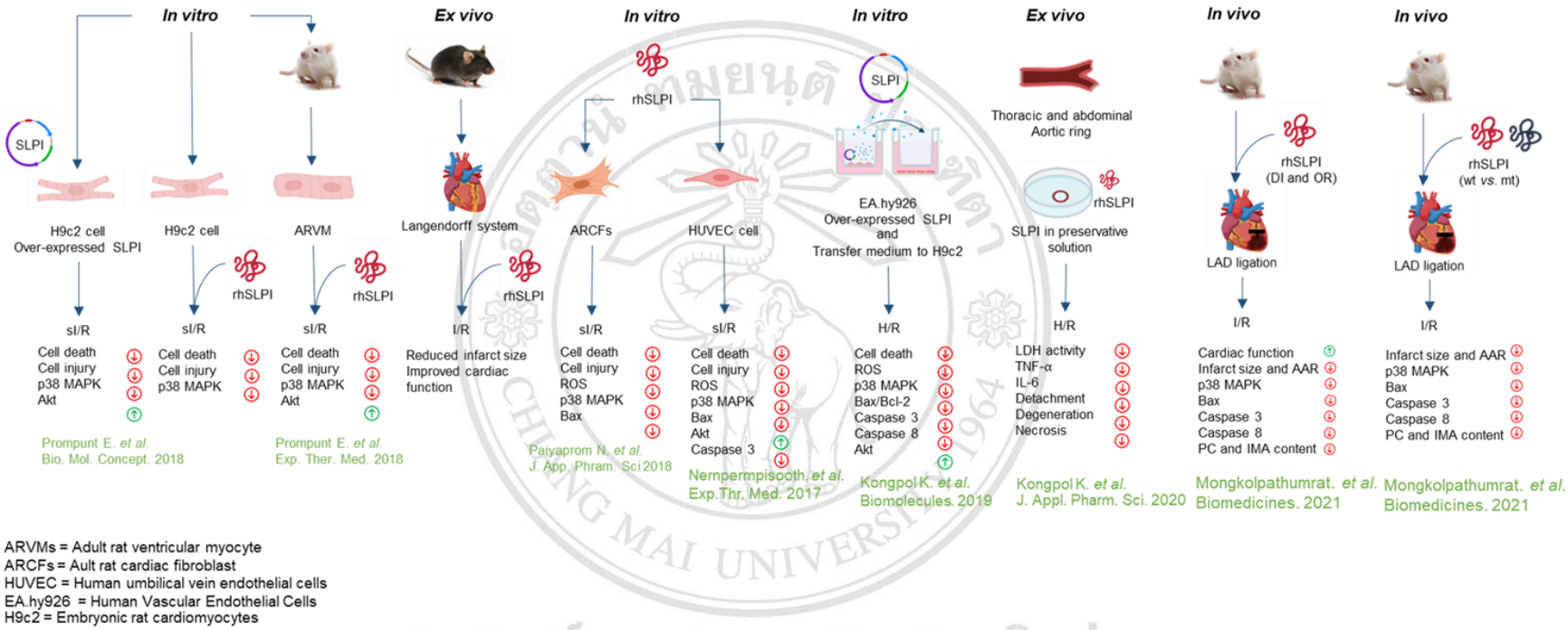


Figure 7 Schematic diagram of previous finding on cardioprotective effects of SLPI.

2.10. Nanoparticles: The definition

In term “nanoparticles” was defined by Richard Adolf Zsigmondy, who used dark field ultramicroscopy which can see particles smaller than a monochromatic light wavelength in 1941 [90]. Generally, the size of nanoparticles is restricted to 1 to 1,000 nm. In addition, physical chemistry is typically used to define nanoparticles since their behaviour depends on a combination of factors that can be measured directly (mass, volume, electric charge), as well as on their propensity to self-organize due to their high reactivity [91].

2.11. Characteristics of nanoparticles

2.11.1. Size of nanoparticles

Size of nanoparticles is important in body response in role of distribution and elimination. In drug delivery, nanoparticles should avoid eliminate from filtration by liver, spleen, or lung and the reticuloendothelial system (RES) [92]. A large size of nanoparticles can be taken up in cells more than a small size of particles because of highly curved surfaces of the large particles more than on small particles [93]. The cellular uptake is highly reliant on particle size because the efficiency of cellular uptake of nanoparticles can be affected by the interaction of the nanoparticles with cells [94].

2.11.2. The polydispersity index (PDI) of nanoparticles

The polydispersity index (PDI), which reflects the spread or width of the particle size distribution, is a crucial parameter. The PDI score actually ranges from 0 to 1, with values greater than 0.7 potentially indicating polydisperse particle size distributions and values less than 0.1 to 0.7 typically implying monodisperse particles [95, 96] (Figure 8).

2.11.3. Surface of nanoparticles

A surface charge of nanoparticles influences a cellular uptake due to high negatively charged nanoparticles show a low cellular uptake when compared to low negatively charged or positively charged of particles with the anionic cell membrane [97]. The factor for cellular uptake of nanoparticles may be the absolute value of zeta potential. Positive or negative surface charges cause an increase in cellular uptake, while nanoparticles with the lowest absolute amount of zeta potential can successfully prevent the absorption [92]. The zeta potential is significant because it is a crucial component of several processes, including electro-osmosis, flocculation, and stability. The zeta potential was developed using electrophoresis, electroosmosis, and streaming, three electrokinetic processes, thanks to advancements in the study of the electrical properties of solid-liquid interfaces [98]. The zeta potential also associates to dispersion of particles are according to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory when electrostatic repulses, triggered by appropriately charged electrical layers around the particles, triumph over the attraction of the Van der Waals interactions [98] (Figure 9).

2.11.4. Shape of nanoparticles

Shape is a crucial factor that influences how quickly and where exactly the system delivers drugs. It can also affect cell absorption. Selecting the right shape for the nanoparticles would enable preferential interaction with particular proteins [99]. Beside of a cellular internalization, adhesion to endothelial cells via cell/particle interactions, and margination to the periphery of blood vessels, shape in addition to other mechanical or biological properties of particles and targeted cells strongly influences the effectiveness of the targeted delivery of nanoparticles in intravascular delivery [92].

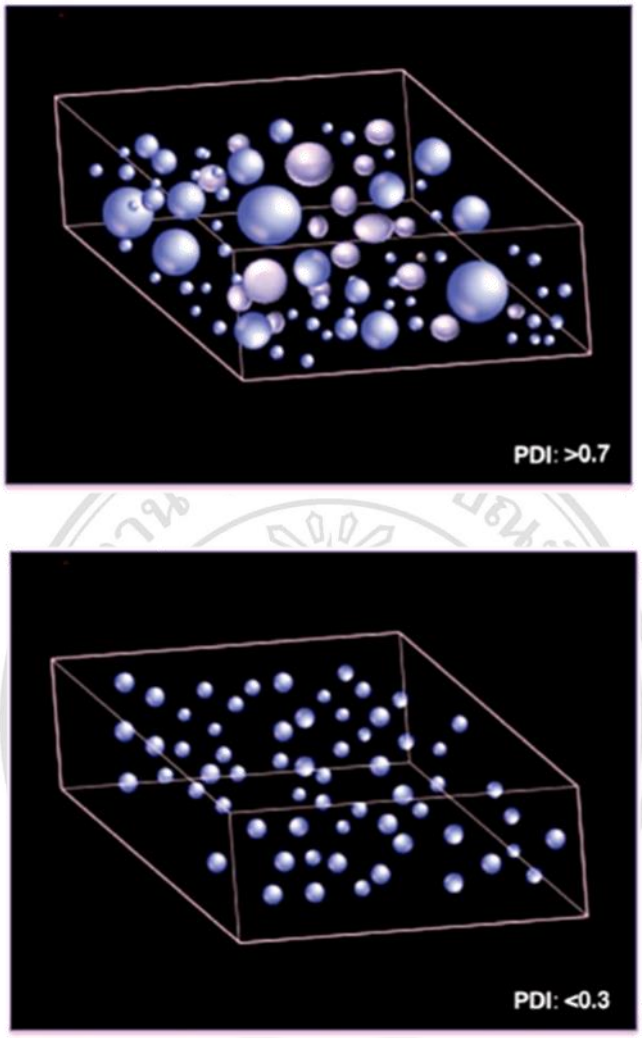


Figure 8 The polydispersity index (PDI) of disperse particles [96].

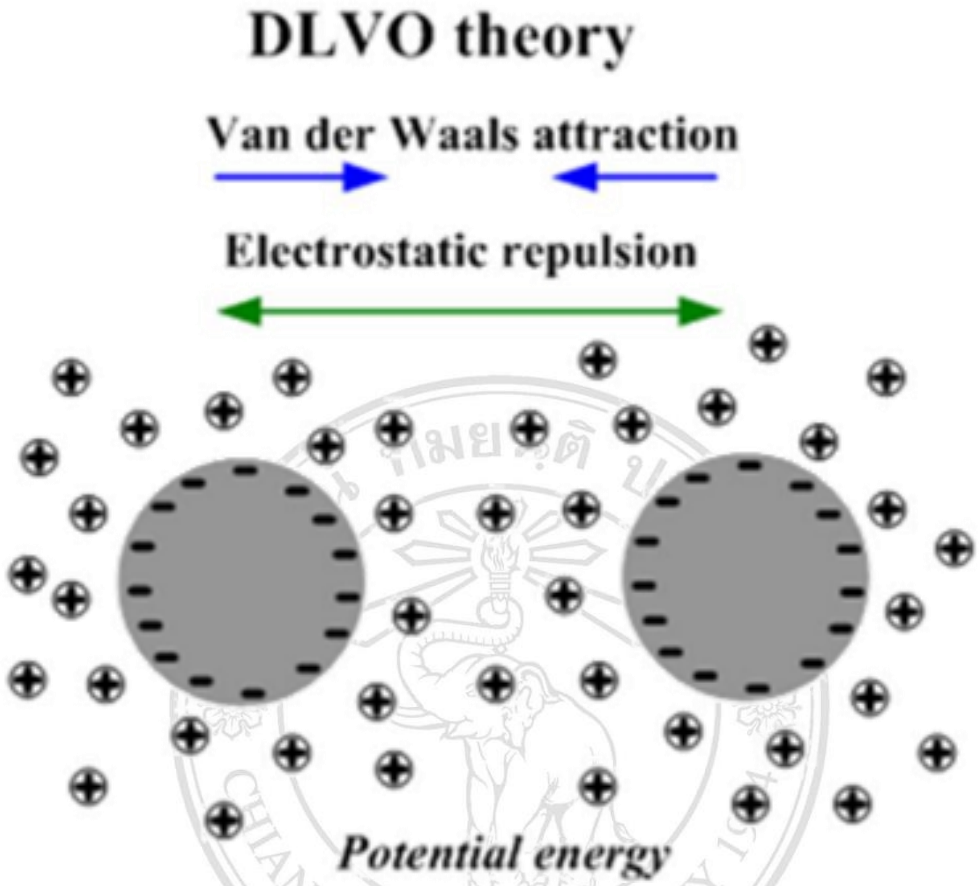


Figure 9 Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. Particles are dispersible when electrostatic repulsion prevails over the Van Der Waals attraction [98].

1.1. Classification of nanoparticles

classification of nanoparticles based on their composition including organic, carbon-based, and inorganic [100-102]. (Figure 10)

1.1.1. Organic nanoparticles

Organic nanoparticles are made from carbohydrates, lipids, proteins, or other organic compounds which are typically non-toxic, and bio-degradable [103]. These nanoparticles are formed by non-covalent intermolecular interactions. As a result, it encourages nanoparticles to provide a pathway for bodily clearance [104]. Nowadays, organic nanoparticles are mostly used in drug delivery in the biomedical field [103] and in transferring a gene for cancer therapy [105].

1.1.2. Carbon-based nanoparticles

Carbon-based nanoparticles are made from carbon atoms [103]. Carbon-based NPs often can be applied in many applications such as bioimaging, energy storage, drug delivery, and monitoring microbial ecology or detecting microbial pathogens [106-109]. Characteristics of carbon-based nanoparticles are biocompatibility and non-toxicity, which are useful for drug delivery and medical applications [110].

1.1.3. Inorganic nanoparticles

Inorganic nanoparticles are made from other compounds except carbon and organic materials. The inorganic materials are metal precursors, which can be monometallic, bimetallic, or polymetallic [111]. Moreover, some metal nanoparticles have special biological, magnetic, and thermal properties [112]. In addition, they become more crucial resources for the creation of nanodevices, which are utilized in a variety of chemical, physical, biological, medicinal, and pharmacological applications [113].

1.2. Nanoparticles for drug delivery

Recently, nanoparticles had the ability to increase the stability and effectiveness of encapsulated cargos by enhancing their stability and solubility, boosting transport across membranes, and lengthening circulation times. In addition, nanoparticles research has been widely conducted, yielding encouraging results *in vitro* and in small animal models [114]. Moreover, in application of drugs with very low solubility have a variety of biopharmaceutical delivery problems *in vivo*. However, all of these restrictions could be removed by incorporating nanotechnology methods into the medication delivery system [115]. The development of an enhanced drug delivery system will be facilitated by nano-based drug delivery systems. There are many types of nanoparticles which could be as drug delivery systems. Chitosan is used for drug delivery to a variety of epithelial types, such as the pulmonary, nasal, intestinal, and buccal epithelium [116-119]. Liposome is used in medication delivery to malignant and tumour tissues [120]. Inorganic nanoparticles, including silver, gold, iron oxide, and silica nanoparticles, are a popular candidate for drug delivery due to high drug loading capacity, variable pore size and volume, ease of functionalization, and biocompatibility [121].

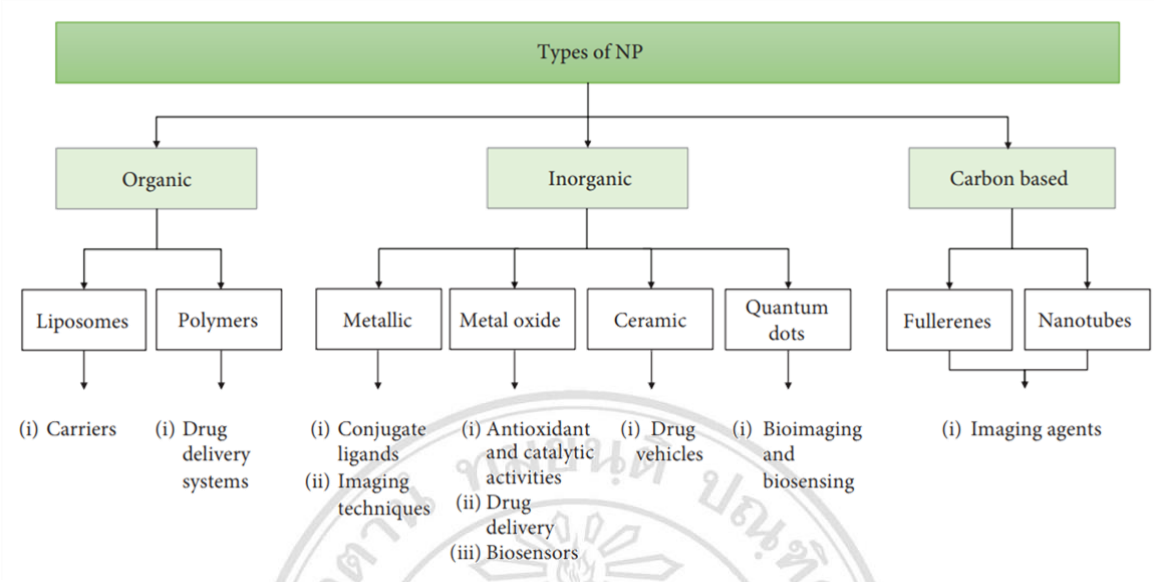


Figure 10 Types of nanoparticles and biomedical applications based on their component [102].

1.3. Nanoparticles encapsulating SLPI

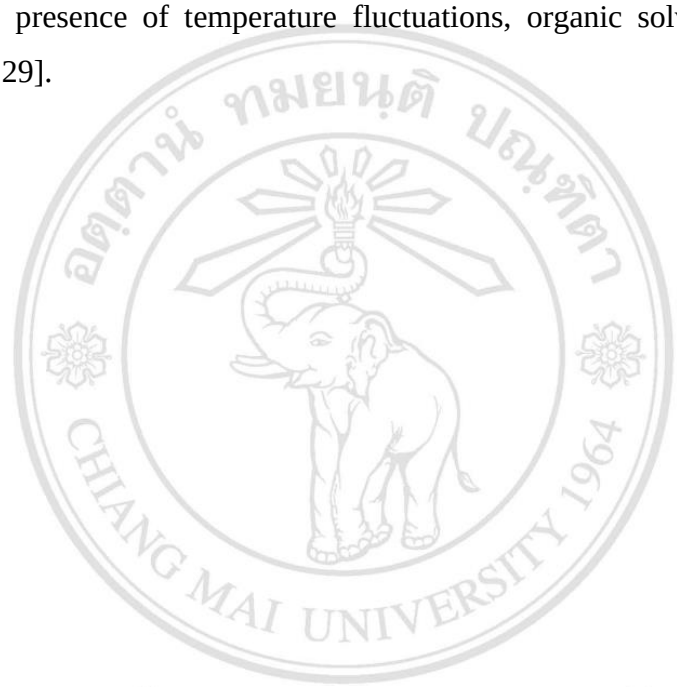
Previous studies have reported 3 types of nanoparticles that contain SLPI (Table 1). To defend against cathepsin L digestion in the lung, Gibbons A. et al. employed liposome nanoparticles to encapsulate SLPI in 2009 [122] . Liposome nanoparticles provide a high SLPI protection against gradation of cathepsin L. In 2011, they used liposome, which encapsulates SLPI in an asthma model. Liposomes provide a protective effect clearance of SLPI in the systemic circulation and produce stable, protective delivery systems for inhalation [123]. In 2019, Hill M. et al. reported alginate/chitosan nanoparticles to deliver SLPI for pulmonary applications by using Tobramycin and SLPI to be antimicrobial and increased the efficacy of drug delivery over prolonged periods [124]. In 2020, Tarhini M. et al. reported human serum albumin (HSA) nanoparticles encapsulating SLPI for antibacterial. It has been demonstrated that SLPI's antibacterial and anti-NE properties are unaffected by its entrapment within HSA nanoparticles [125].

Table 1 previous studies of SLPI encapsulated nanoparticles

Nanoparticles	Conc. SLPI (mg/ml)	Size of NPs (nm)	Applications	References
Liposome	0.33	153.6	Asthma model	[123]
Liposome	0.001	200	Protection of SLPI from enzyme digestion	[122]
Alginate/Chitosan	25	437.5	Antibacterial and antiinflammation	[124]
HSA	1.5	127	Antibacterial activity	[125]

1.4. Silica nanoparticles

Silica nanoparticles are candidates for drug delivery because silica is the most abundant compound on Earth, silica is found in plants and cereals and is widely distributed throughout the planet's crust [126]. The surface area, particle size, and pore size of silica nanoparticles can be adjusted, and they have good biocompatibility [127]. Therefore, these characteristics make the inorganic silica more stable than traditional drug delivery techniques in the presence of temperature fluctuations, organic solvents, and acidic conditions [128, 129].



1.5. Synthetic methods of silica nanoparticles

1.5.1. Non-porous silica nanoparticles

Non-porous silica nanoparticles are a type of silica nanoparticles without a distinctive structural shape and irregular form. They are frequently used in therapeutics, imaging, enzyme encapsulation, drug delivery, and other applications because of their exceptional biocompatibility [129, 130].

1) Thermal method

Burning $SiCl_4$ at a flame temperature of more than 1000 °C in an oxygen-rich hydrogen gas flame reactor created a fume of SiO_2, which was manufactured using the thermal technique [129].

2) Precipitation method

A weak acid is used to acidify an alkali metal silicate solution in order to produce a gelatinous precipitate. After filtering, washing, drying, and grinding this precipitate are colourless silica particles [129, 131].

3) Chemical sol-gel method

Using colloidal particles in a sol or alkoxide precursors in water can create a hydrosol, the sol-gel technique creates a highly amorphous gel product in solution. The change from a solution to a gel is brought about by a rise in viscosity, which leads the entire gel to change into a solid state [132].

4) Stöber synthesis

Monodisperse silica particles are created utilizing this physicochemical method. In this method, a variety of sol-gels are formed by hydrolysing and condensing metallic salts, metal alkoxides made by adding alcohol, or other organometallic silicon precursors [131]. In the reaction, alkyl silicates act as a hydrolysing agent, and the result is condensed in alcohol solutions using silicic acid. Ammonia serves as the process's catalyst. The produced sol-gel is removed, cleaned, and dried after centrifugation [129]. (Figure 11)

1.5.2. Mesoporous silica nanoparticles

Mesoporous silica nanoparticles have exceptional physicochemical qualities, including a high surface area, and unique structural features. One can adjust the pore aperture size and environment to reserve particular molecules of interest [133]. Consequently, mesoporous silica nanoparticles are often used in bioimaging, drug delivery, and catalysis [129]. Improved Stöber synthesis was modified from the Stöber process to make porous silica by using a surfactant template, which includes cetrimonium bromide (CTAB) [134], cetyltrimethylammonium chloride, and glycerol [135]. The surfactant forms micelles, which are a hydrophobic interior and a hydrophilic surface producing particles with surfactant- and solvent-filled channels. Porous silica was made by burning or evaporating the surfactant and solvent molecules during the calcining process, mesopore gaps are left throughout the structure [121, 136]. (Figure 11)

1.6. Optimization of Nanoparticles

he improvement of the capacity of drug into inorganic nanoparticles is main considering due to high surface area and size of nanoparticles. The benefit of an inorganic carrier nano-drug delivery method is enhancing drug targeting, and contemporary inorganic materials include NPs based on silica [137]. Silica nanoparticles have been improved by Andreani et al. using a variety of hydrophilic polymer coatings, including chitosan, sodium alginate, and low- and high-molecular-weight polyethylene glycol. This transports oral insulin by mucosal adhesion. Using a protein coating that has a high affinity for hydrophilic polymer chains, it was demonstrated that insulin can attach to silica nanoparticles more than 90% [137, 138]. In order to produce a pH response, Peng et al. synthesised a core-shell hybrid silica nanoparticle made of a copolymer shell made of N-(3,4 dihydroxyphenethyl) methacrylamide (DMA) and N-isopropylacrylamide (NIPAM). Moreover, poly (Nisopropylacrylamide) can improve the material's sensitivity to temperature. The drug loading was increased from 8.6% to 28.0% by altering the DOX concentration [139].

1.7. Degradability and clearance of silica nanoparticles

Silica nanomaterials can be broken down in aqueous media in just 24 hours or over the course of several weeks depend on their physicochemical characteristics [140]. The degradation process of silica nanoparticles has three steps including hydration, hydrolysis, and ion-exchange processes [140]. Firstly, water is adsorbed into silica conjugated structure, siloxane flamework. After that, siloxane is hydrolysed to from silanols. Finally, ion-exchange processes lead to the leaching of silicic acid, which consist of nucleophilic attack of OH^- [141] (Figure 12). Silica in forms of silicic acid or polysilicon acid are diffused through the bloodstream or lymph to eventually be eliminated in the urine [142]. In addition, the previous study showed degradation of mesoporous silica nanoparticle (MSN) which size is 80 nm can degrade depending on over time in PBS [142] (Figure 13) and can disappear in plasma, and urine within 72 h (Figure 14).

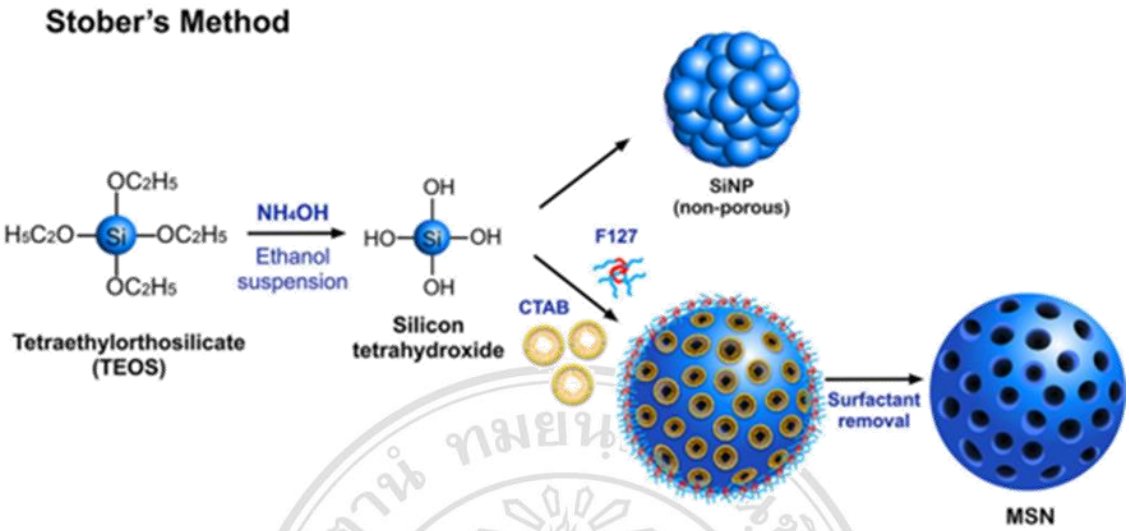


Figure 11 Stöber method to fabricate non-porous silica nanoparticles and mesoporous silica nanoparticles [121].

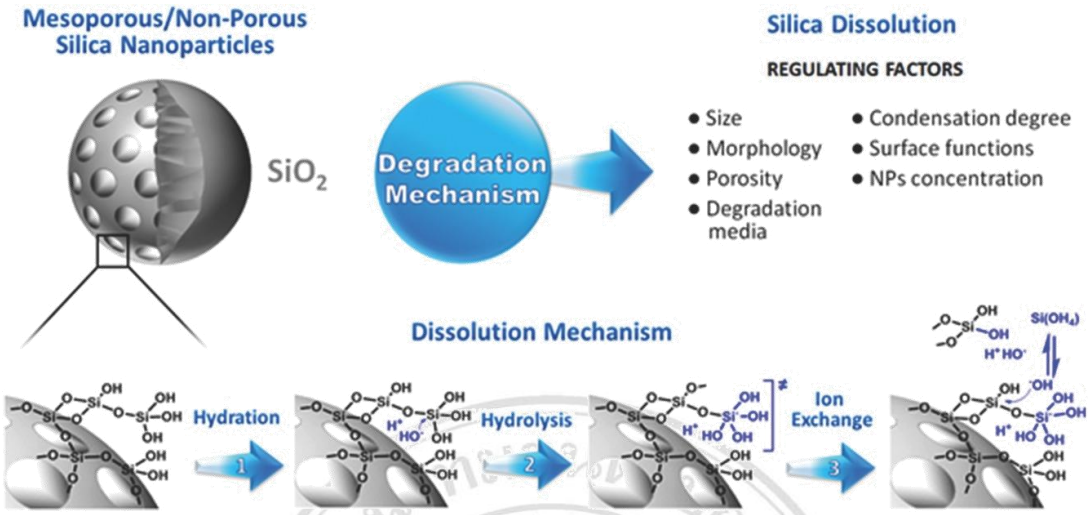


Figure 12 Mechanisms and regulating factors of silica degradation [142].

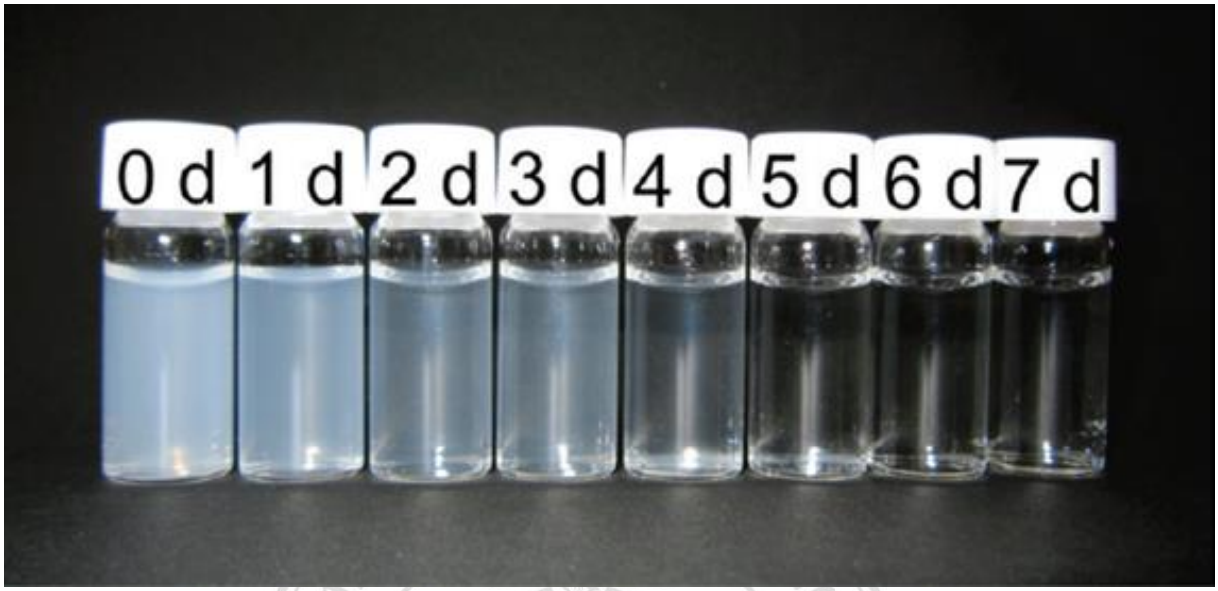


Figure 13 The degradation of silica nanoparticles over time in PBS [143].

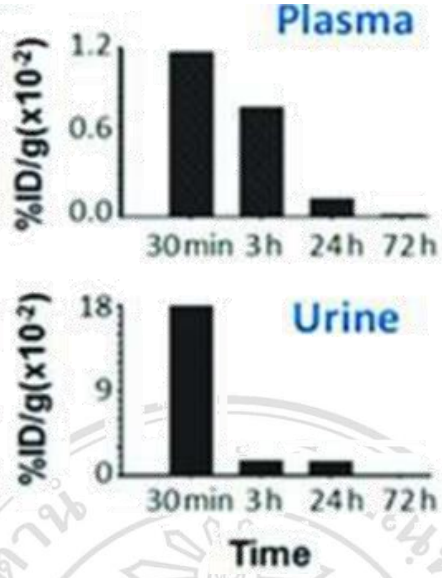


Figure 14 degradation of silica nanoparticles in plasma and urine.

1.8. Gelatine covered silica nanoparticles

Gelatine is a naturally occurring polymer that is partially hydrolysed from collagen. Desirable characteristics of collagen include its natural origin, low cost, low toxicity, biodegradability, and lack of immunogenicity. Gelatine could adhere to the nanoparticle surfaces through intricate interactions like van der Waals, hydrophobic, and electrostatic interactions [144]. Thus, gelatine is a good protein that can be used to control drug release by pH-dependent property. Luo Z. et al. used gelatine to cover mesoporous silica nanoparticles for reducing immunotoxicity. Gelatine-covered MSN can reduce macrophages activating compared to bare silica nanoparticles [18]. Z. Zou. et al. used gelatine to cover MSN for intracellular acid-activated drug delivery. The gelatine coatings can trigger the release of the encapsulated DOX when slightly acidic conditions are present, but they can be released from MSN when the conditions are physiological [145] (Figure 15). Y.-T. Liao. et al. used gelatine to cover MSN for intracameral pharmacotherapy of glaucoma [146] (Figure 16). Thus, protected peptides from protease in blood circulation, silica nanoparticles maybe benefit from being covered in gelatine.

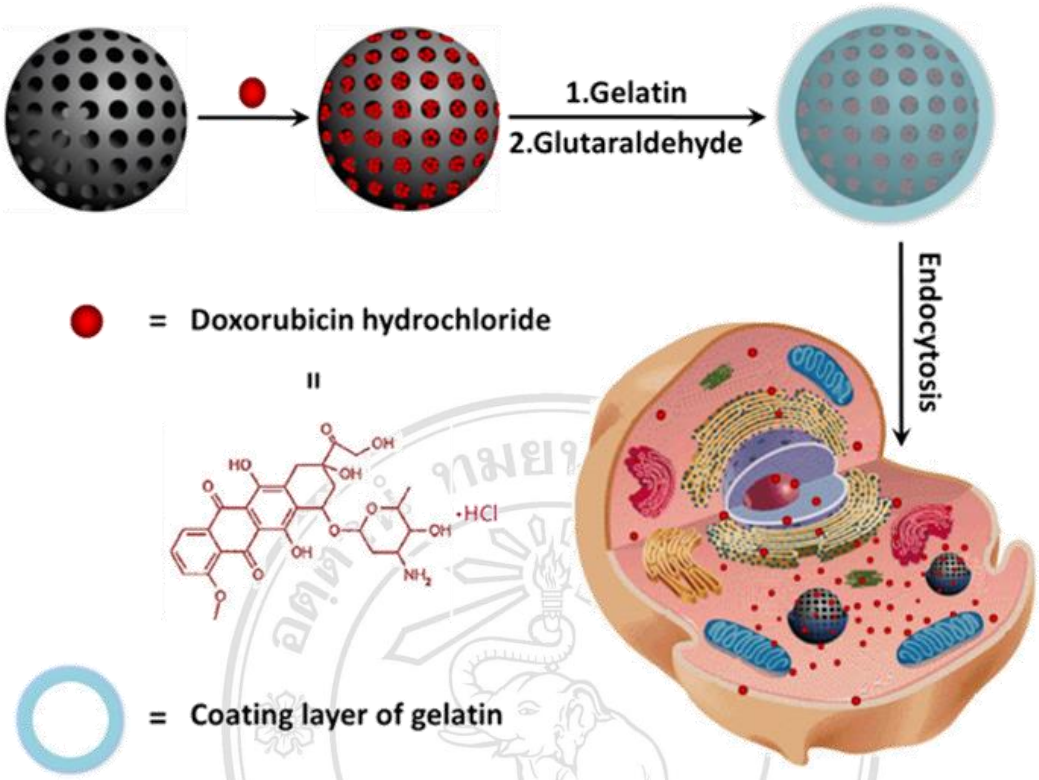


Figure 15 Gelatine used to cover mesoporous silica nanoparticles for delivering Doxorubicin into hepatic cells [145].

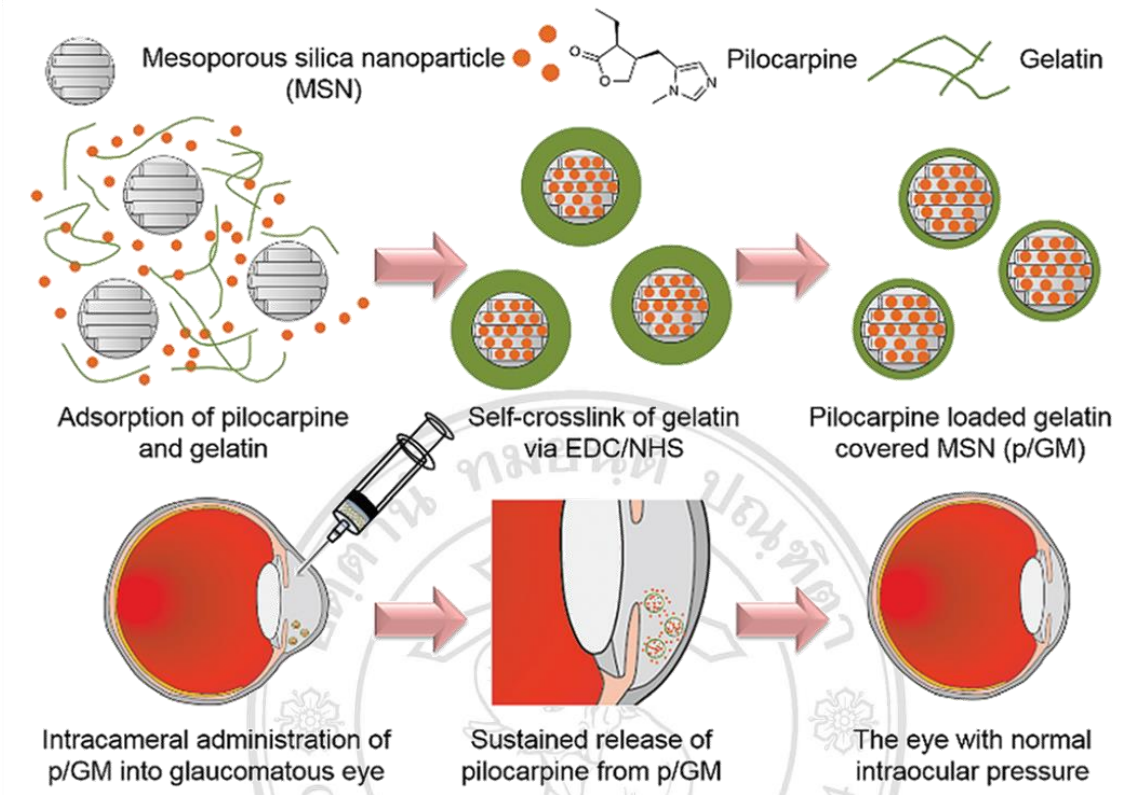


Figure 16 Gelatine used to cover mesoporous silica nanoparticles for delivering Pilocarpine to rabbit's eyes under glaucoma [146].

1.9. Rationale of thesis

Myocardial ischaemia reperfusion injury is a serious condition. One of I/R injury causes contribute to protease, which promotes and aggravates myocardial injury [41]. Inhibition of protease might reduce cardiac cell injury. A protease inhibitor as chymase inhibitor was studied to be potential against I/R injury. However, there was a report that some small, synthesized protease inhibitor showed off-targets [9], and the adverse drug effects is a concern. Thus, using endogenous protease inhibitor protein, which is friendly protein in human body could provide cardioprotection and be safe.

The secretory leukocyte protease inhibitor (SLPI) exhibit cytoprotective effects to protect cardiomyocytes from I/R injury by reducing intracellular ROS production and p38 MAPK phosphorylation and inducing activation of Akt phosphorylation [86]. SLPI can inhibit NE that is released from neutrophils that induce myocardial cell death and injury [85]. Moreover, SLPI can reduce infarct size, and inflammatory cytokines, and oxidatively modified proteins, and apoptosis regulatory signaling, as well as improve cardiac function [89].

The limitation of the biomedical application of SLPI is that SLPI could normally be eliminated through glomerular filtration, then reabsorption and degradation in tubular cells within 2-6 h interval, the estimated half-life of SLPI in plasma is 120 minutes [16]. Besides, SLPI could be inactivated by enzymes that are secreted from the respiratory tract such as cathepsin [17]. Thus, the transportation of SLPI to the target organ is a new challenge. Therefore, any strategies to improve the stability and extend the half-life of SLPI in the system could provide greater therapeutic benefit.

Several advantages of nanoparticles for peptide drug carriers have been listed, for example reducing the enzymatic digestion and aggregation of peptide drugs, and increasing the transmembrane absorption. The nanoparticles that loaded rhSLPI are used in the asthma model, they exhibit a protection of SLPI from enzyme digestion,

antibacterial and anti-inflammation, and anti-bacterial activity. However, the effect of nanoparticles that loaded rhSLPI in cardiovascular system has never been determined.

Therefore, in this thesis, rhSLPI was encapsulated in gelatine-covered silica nanoparticles and will be determined for its therapeutic potential against ischaemia/reperfusion (sI/R) injury in rat cardiomyocytes (H9c2 cell line).

CHAPTER 3

Research Methodology

This chapter presents the methodology of this research including material and method. The detail of each topic is described below.

The conceptual idea of experiment is to fabricate the gelatine-covered silica nanoparticles (GSNPs) for delivering recombinant human secretory leukocyte protease inhibitor (rhSLPI) (Figure 17) and test for an *in vitro* cardioprotective effect of the fabricated nanoparticles on simulated ischaemia/reperfusion injury in rat cardiac myoblast (H9c2) cell line. (Figure 18)

In this master's degree thesis, the study was divided into 2 major parts according to the aim of study.

Aim 1 to prepare gelatine covered silica nanoparticles to encapsulating rhSLPI (rhSLPI-GSNPs)

The rhSLPI loaded nanoparticles was prepared base on gelatine-covered silica nanoparticles. Then, the physical properties of rhSLPI functionalized nanoparticles were determined the morphology by Scanning electron microscope (SEM), the zeta potential and polydispersity index (PDI) were measured by Zetasizer analyser, and the sizes of nanoparticles were measured by using ImageJ software. The quantification of rhSLPI in encapsulated nanoparticles and released form were also measured.

Aim 2. To determine cardioprotective effect of rhSLPI loaded gelatine covered silica nanoparticles (rhSLPI-GSNPs)

The cytotoxicity and cardioprotective effect of rhSLPI-loaded nanoparticles on H9c2 cells were subjected to an *in vitro* simulated ischaemia/reperfusion (sI/R). The cardioprotection was determined via a percentage of cell viability by MTT viability assay and cellular injury by released- lactate dehydrogenase (LDH) activity assay.

2.1. Chemicals and Reagents

The chemicals were purchased from Sigma–Aldrich including Tetraethyl orthosilicate (TEOS) (98.0%), Gelatine type A, Glutaraldehyde, 30% ammonia solution (NH_4OH), and ethyl alcohol. Recombinant human SLPI (rhSLPI) was purchased from Sino Biology Inc (Beijing, China). In cell culture, reagents were purchased from Gibco (Gibco BRL; Life Technologies Inc. New York, USA) including Dulbecco's modified Eagle's medium (DMEM), Foetal bovine serum, penicillin, streptomycin and trypsin-EDTA. 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Ameresco (Solon, Ohio, USA). CyQUANT™ LDH Cytotoxicity Assay Kit (Cat No: C20301) was purchased from Thermo Fisher.

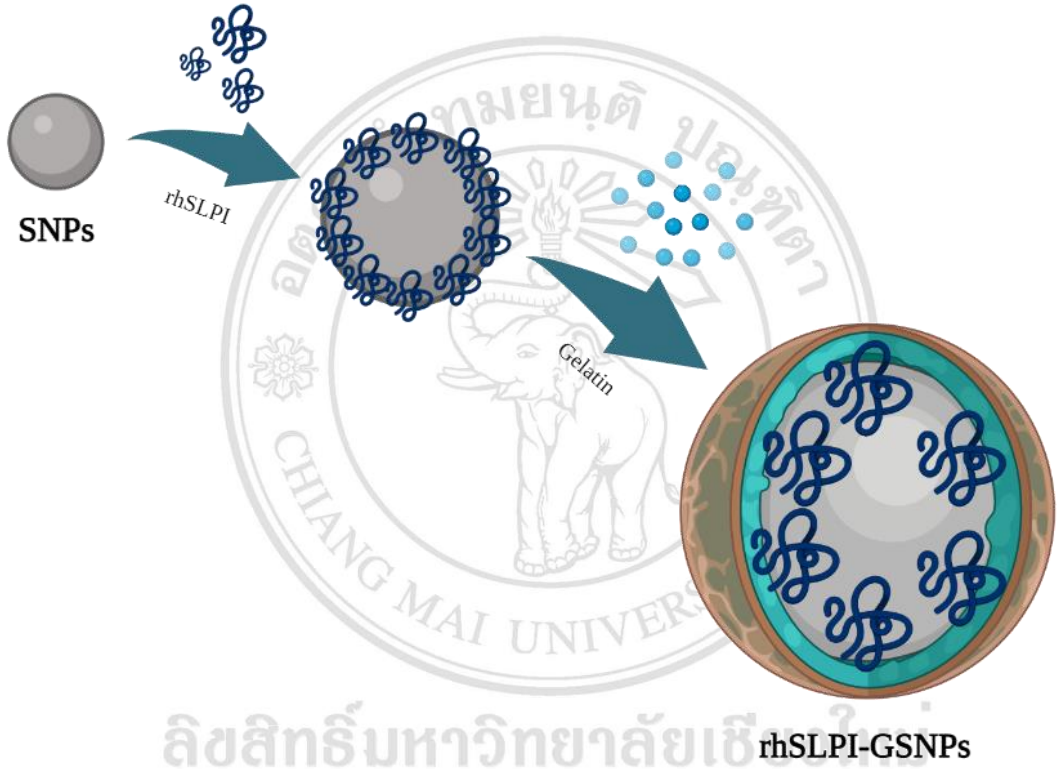


Figure 17 The rhSLPI-GSNPs model. The rhSLPI bind on surface of silica nanoparticles (SNPs) and was covered with gelatine for protecting rhSLPI from protease digestion.

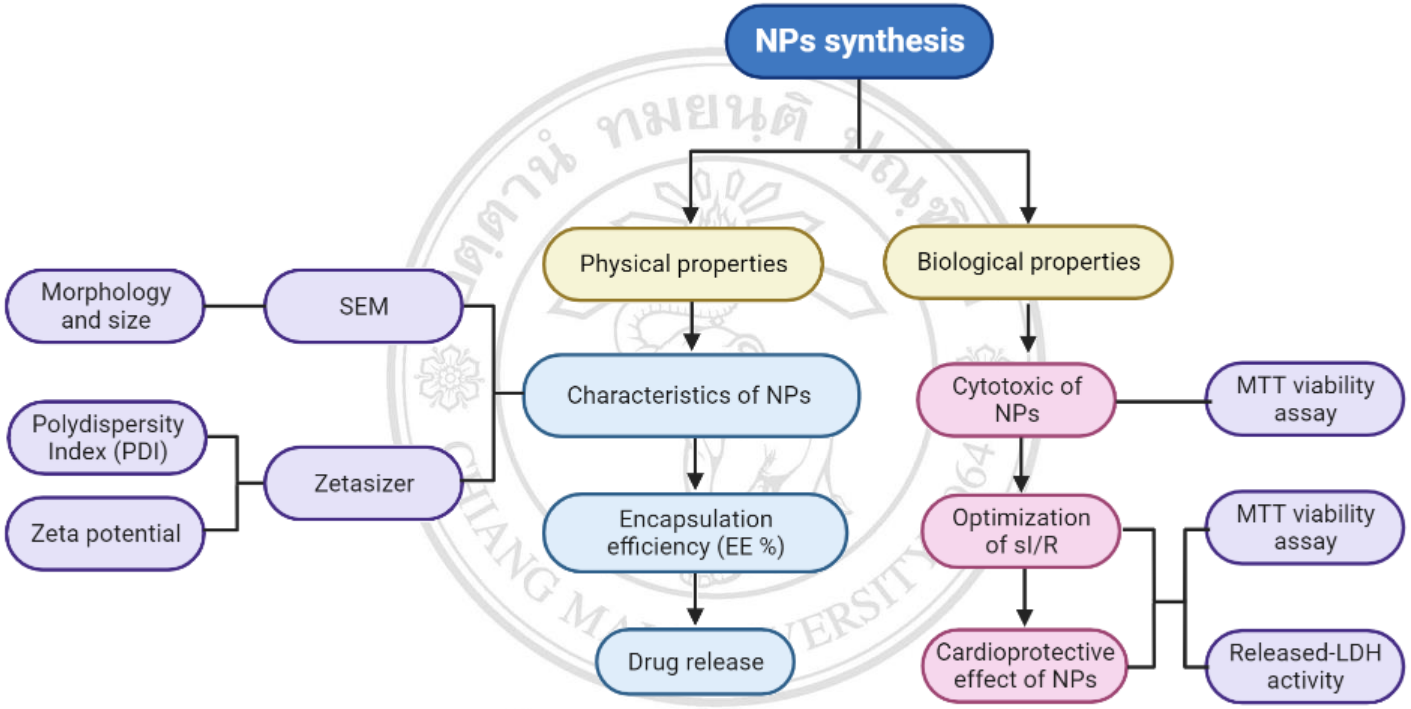


Figure 18 The experimental design. The experimental design for aim 1, Preparation of nanoparticles and determination of physical properties. The experimental design for aim 2, *in vitro* cellular effects on cardiac cells.

2.2. Cell and cell culture

The H9c2 cell is derived from embryonic BDIX rat heart tissue and shown the feature of skeletal muscle. It could express nicotinic receptors and generate phosphokinase isoenzyme [147]. Moreover, previous study reported that the H9c2 cell showed morphological characteristics similar to cardiomyoblast and showed the biological properties of adult cardiac cells [148]. Thus, this cell line is appropriate as an in vitro model for cardiac muscle. The H9c2 cell was purchased from American Type Cell Culture (ATCC-CRL1446). The H9c2 cell was cultured in complete DMEM medium, which contained with 5000 units/mL of penicillin, 5000 µg/mL of streptomycin and 10% foetal Bovine Serum (FBS). The cell was cultured at 37 ^{o}C, 5% carbon dioxide (CO_2) and 95% air during the entire experiment. When the cells density reaches 70-80% confluent, the cell was sub-cultured. The cell was washed with 5 mL pre-warmed phosphate buffer saline (PBS) twice and then the culture media was aspirated from cell culture flask.

After that, 2 mL of pre-warmed trypsin-EDTA (Gibco®) was added and incubated at 37 ^{o}C for approximately 2 min or when observe under microscope the cell detached from the flask surface. Then, the trypsin activity was terminated by adding 5 mL of pre-warmed complete DMEM medium. The cell suspension was transferred into 15 mL sterile tube and centrifuged at 125 x g for 5 min. The supernatant was removed, and the cell pellet was resuspended with 1 mL of complete DMEM medium. The cell was transferred into a new cell culture flask and the flask was added at least 9 mL of complete DMEM medium.

2.3. Preparation of gelatine-covered silica nanoparticles-encapsulated recombinant secretory leukocyte protease inhibitor (rhSLPI-GSNPs)

Silica nanoparticles (SNPs) were synthesized by a modified Stöber process as described in the previous study [149]. Briefly, a co-solvent containing distilled water and ethanol was added to a round flask. Then, TEOS (1 mL) was added to the solution. The solution was stirred and NH_4OH slowly dropped for the chemical reaction in 8 hrs. Then, the nanoparticles were centrifuged and washed three times with distilled water and ethanol. The rhSLPI-loaded gelatine covered silica nanoparticles (rhSLPI-GSNPs) were prepared by using glutaraldehyde crosslinking [145] (Figure 19). SNPs were added in 500 µL of rhSLPI solution (0.33 mg/mL) following the previous study (113) and mixed overnight. Then, the solution was centrifuged to remove excess rhSLPI. Pellets were stirred in gelatine solution (1%, 1 mL) at 40 °C for 6 hrs. After that, cold deionized water (4 °C) was added to the mixture. Then, 1% glutaraldehyde solution was added to use for cross-linking the gelatine for 8 hrs. The solution was centrifuged to collect the particles and washed 3 times with distilled water. Finally, the rhSLPI-GSNPs were dried by freeze-drying and keep in a desiccator for the next use (Figure 20).

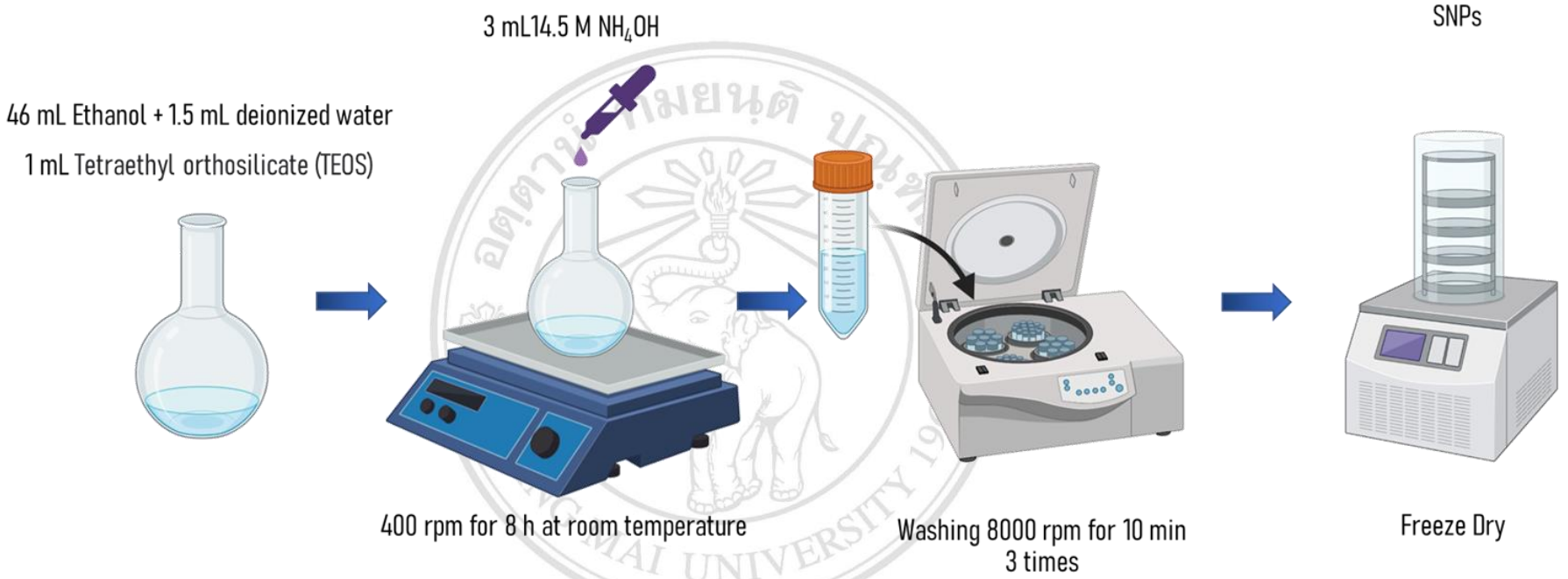


Figure 19 Schematic diagram demonstrating the fabrication of silica nanoparticles (SNPs) by Stöber process.

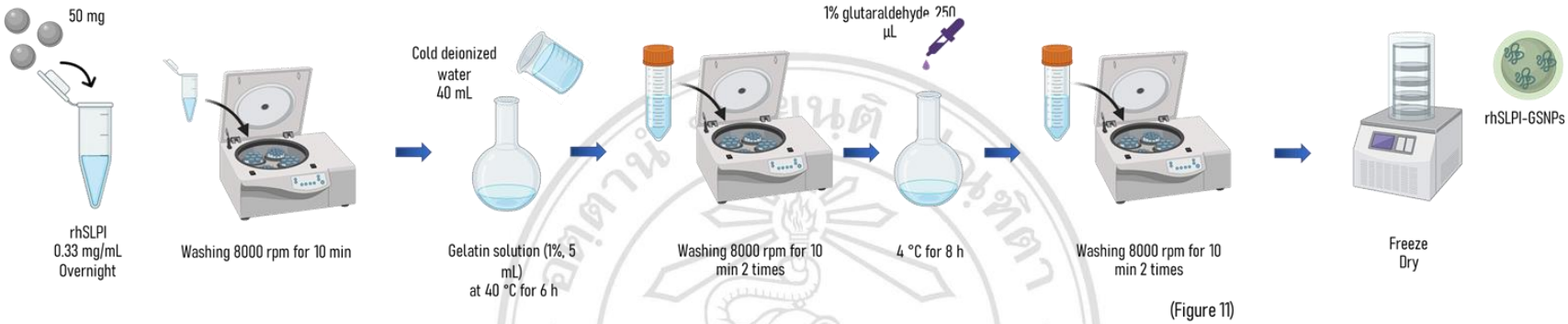


Figure 20 Schematic diagram demonstrating the fabrication of rhSLPI-gelatin covered silica nanoparticles (rhSLPI-GSNPs) .

2.4. Physical characterizations of rhSLPI-GSNPs

2.4.1. Determination of nanoparticle morphology by field emission scanning electron microscopy (FE-SEM)

Each nanoparticle was dissolved with DI water and dropped on copper tape. Then, allow the copper tape to air dry. The copper tape was sticked on the basement (Stub) (Figure 22). After that, basement was coated with platinum for 3 nm thickness. Finally, the morphology of nanoparticles was determined by field emission scanning electron microscopy (FE-SEM) (CLARA, TESCAN) at Maejo University.

2.4.2. Determination of particle size and polydispersity Index (PDI)

The size of each nanoparticle was measured by ImageJ software (National Institute of Health) from figures of SEM, at Maejo University. The nanoparticles were dissolved with DI water. The sample was poured in the disposable plastic micro cuvette (ZEN0040) (Figure 21b) for measuring PDI of nanoparticles. PDI was measured by Zetasizer (Malvern, England) (Figure 21a) at Faculty of Pharmacy, Chiang Mai University.

2.4.3. Determination of electrical property of nanoparticles

The nanoparticles were dissolved in DI water. The sample was poured in the folded capillary cell (DTS1070), which is disposable cuvettes primarily for the measurement of zeta potential (Figure 21c) for measuring zeta potential of nanoparticles. the zeta potential was measured by Zetasizer (Malvern, England) (Figure 21a) at Faculty of Pharmacy, Chiang Mai University.

a

b

c

Figure 21 Equipment of Zetasizer. a) Particle size analyser Malvern (Zetasizer ZS) b) Disposable plastic micro cuvette (ZEN0040) for measuring PDI and size of nanoparticles. c) folded capillary cell (DTS1070) for measuring zeta potential of nanoparticles.

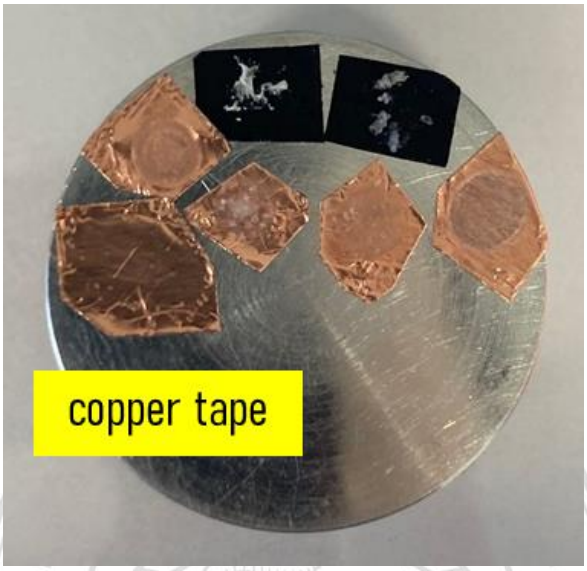


Figure 22 Stub for scanning electron microscopy (SEM). Samples were dropped on copper tape, which sticked on stub.

2.5. Quantification of encapsulated SLPI

The 0.5 mg of rhSLPI-GSNPs was dissolved with 1 mL of 0.1% collagenase. Then, rhSLPI-GSNPs was incubated at 37 ºC for 4 hrs for breaking gelatine that was covering on nanoparticles. the rhSLPI was determined by ELISA.

The encapsulation efficiency was calculated using the following formula:

$$\text{Encapsulation efficiency} = \frac{\text{Amount of drug encapsulated}}{\text{Total drug}} \times 100$$

2.6. Drug release

Drug releases were performed in two experiments including Phosphate Buffered Saline (PBS) solution, and 0.1% collagenase solution. First, 5 mg of rhSLPI-GSNPs were dissolved in 1 ml of PBS. Releasing time in PBS was separated to 0, 24, 48, and 72 hrs respectively at 37 ºC with shaking at 400 revolutions per minute (rpm). rhSLPI-GSNPs-PBS was centrifuged at 8000 rpm for 10 min. After that, 100 µL of supernatant was corrected and PBS was added for retaining rhSLPI-GSNPs concentration. Second, 5 mg of rhSLPI-GSNPs were dissolved in 1 ml of 0.1% collagenase solution. Releasing time in 0.1% collagenase was performed to 0, 15, 30, 60, 120, and 240 min at 37 ºC with shaking at 400 rpm. rhSLPI-GSNPs-collagenase was centrifuged at 8000 rpm for 10 min. After that, 100 µL of supernatant was corrected and 0.1% collagenase was added for retaining rhSLPI-GSNPs concentration. Supernatants were diluted 1:10 with DI water and measured SLPI concentration by ELISA. SLPI concentration was calculated to be a cumulative percent release that was compared by using EE% as 100%.

2.7. Determination of SLPI by enzyme-linked immunosorbent assay (ELISA)

The SLPI concentration in samples was measured by ELISA kit (sandwich ELISA Quantikine®), R&D Systems, Inc, USA. The diluents RD1Q was added into a microplate, which was coated with the monoclonal antibody specific for SLPI protein and incubated at room temperature for 2 hrs. Then, the excess solution was discarded, and was washed by washing buffer. After that, detection antibody, conjugated with horseradish peroxidase (HRP), was added into wells and incubated for 2 hrs. Then, the microplate was washed three times by washing buffer for removing the unbounded detection antibodies. Then, HRP substrates was added to each well and incubated for 20 min in the dark room. Then, stopped solution was added into wells. The colour of solution was changed from blue to yellow, which was determined by using spectrophotometer at O.D. 450 nm. The measured value of O.D. was calculated with standard curve of SLPI concentration (pg/mL).

2.8. Determination of cytotoxicity of rhSLPI-GSNPs

Cytotoxicity assay was performed by using MTT viability assay. First, the H9c2 cell was seeded to 96-well plate at 1×10^5 cells/well in complete DMEM medium, which supplemented 5000 units/mL of penicillin, 5000 µg/mL of streptomycin and 10% Festal Bovine Serum (FBS). Then, the cell was incubated with concentrations of GSNPs and rhSLPI-GSNPs 0, 0.1, 1, 10, and 100 µg/mL for 24 hrs at 37 °C. After that, the complete Dulbecco's Modified Eagle Medium (DMEM) was discarded, and cell viability was determined by MTT cell viability assay.

2.9. MTT cell viability assay

2.9.1. Principle of MTT viability assay

MTT viability assay is the enzyme-base method by using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT). MTT will be transformed to formazan by mitochondrial succinate dehydrogenase enzyme (dark purple) (Figure 23). The formazan will be solubilized with an organic solvent for instance dimethyl sulfoxide (DMSO) then measure absorbance by spectrophotometer [10, 122, 150].

2.9.2. Determination of cell viability

Cells were cultured and exposed to stimuli or toxic agent. At the end of incubation period, the culture medium was discarded and MTT reagent (0.5 mg/mL in PBS) was added and incubated at 37°C for 2 h. Then, MTT reagent was discarded, and DMSO was used to solubilize the formazan dye. The colour of solution was determined by spectrophotometer at O.D. 570 using DMSO as a blank. The difference in cell death was calculated as relative percentage of cell viability.

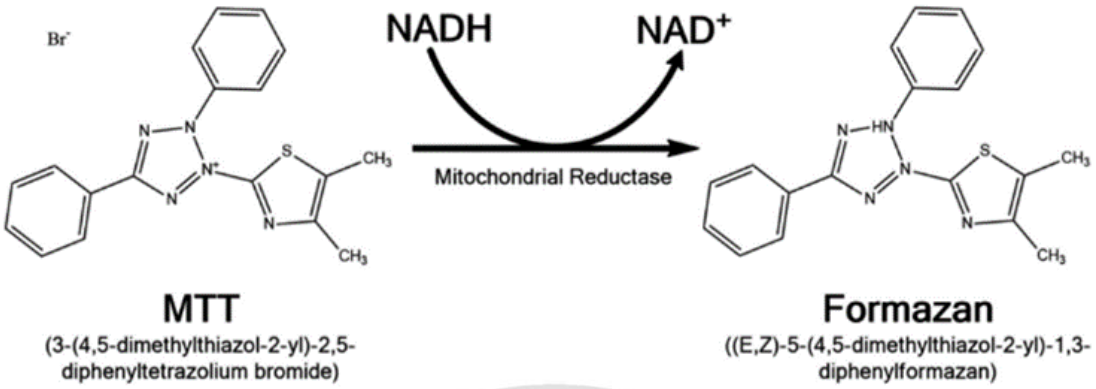


Figure 23 Reduction of MTT to formazan crystals [150]

2.10. Determination of cellular injury by released-lactate dehydrogenase activity

2.10.1. Principle of lactate dehydrogenase (LDH) activity assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is found in all cells. LDH is quickly released into the cell culture supernatant when the plasma membrane is disrupted, a crucial aspect of cells going through apoptosis, necrosis, and other types of cellular destruction. By reducing a second chemical in a linked reaction to a substance with easily quantifiable features, the NADH generated during the conversion of lactate to pyruvate can be used to measure the released-LDH activity [151].

2.10.2. Method for determining released-LDH activity

Transfer 50 µL of each sample medium, which collected before MTT assay to 96-well flat-bottom plate. Then, 50 µL of Reaction Mixture was added to each sample and mixed well. The plate was incubated at room temperature for 30 minutes in dark room. The stop solution was added to each sample well, then mix by gentle tapping, which did not have any bubbles present in wells. Measure the absorbance at O.D. 490 nm and 680 nm. Result was interpreted by O.D. 490 minus 680.

2.11. Optimization of simulated I/R injury (sI/R) conditions

2.11.1. Principle of simulated ischaemia

Simulated ischaemia (sI) was performed by following previous studies [12, 41]. Simulated ischaemia method has demonstrated the clinical condition, which showed some characteristics of ischaemia. The ischaemia is performed by inducing tissue to be low hypoxia, which is a cause of adenosine triphosphate (ATP) reduction in myocardial ischaemia [152]. *In vitro* studies have attempted to simulate ischaemic conditions. The hypoxia was simulated by using sodium dithionite [153], which capture oxygen. The anaerobic glycolysis was simulated by using 2-deoxyglucose, which is a nonmetabolizable glucose analogue for shutdown cellular metabolism [154]. Intracellular acidosis was simulated by using sodium lactate. All of condition is made metabolic blockage.

2.11.2. Optimization of simulated ischaemia timings

The H9c2 cell was seeded to a 96-well plate at 1×10^5 cells/well in complete DMEM medium, which contained 5000 units/mL of penicillin, 5000 µg/mL of streptomycin, and 10% foetal Bovine Serum (FBS). For optimization of sI/R duration that causes 50% cardiac cell injury, cells were subjected to sI/R by the ischaemic buffer, which contains 3.8 mM KCl, 0.49 mM $MgCl_2$, 137 mM NaCl, 0.9 mM $CaCl_2$, 4.0 mM HEPES (containing 20 mM 2-deoxyglucose), 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5. The duration time of treatment was separated into 10, 20, 30, 40, 50, and 60 mins at 37°C [155, 156]. After that, the ischaemic buffer was discarded. The complete DMEM medium was added for reperfusion for 24 h at 37°C, 5% CO_2. After that, the cell viability was measured by cytotoxicity by MTT cell viability assay. The released LDH activity was determined from the collected media using the LDH Cytotoxicity Assay Kit. Fifty microliter of complete DMEM medium was mixed with 50 µL of Reaction Mixture. The plate was incubated at room temperature for 30 minutes in a dark room. Then, the stop solution was added to the action. LDH activity was measured by the absorbance at 490 nm and 680 nm.

2.12. Determination of the cardioprotective effect of rhSLPI-GSNPs in sI/R injury

The cardioprotective effect of rhSLPI-GSNPs was determined by MTT viability assay. The H9c2 cell was seeded to a 96-well plate at 1×10^5 cells/well in a complete DMEM medium, which contained 5000 units/mL of penicillin, and 5000 µg/mL of streptomycin. and 10% foetal Bovine Serum (FBS). Then, the cell was treated with rhSLPI-GSNPs 1, 10, and 100 µg/mL for 24 h at 37 °C. Then, the complete DMEM medium was removed, and performed sI/R injury was following optimization of sI/R. Then, the complete DMEM medium was removed. The MTT reagent (0.5 mg/mL) was added and incubated at 37°C for 2 h. After incubation, the MTT reagent was discarded, and DMSO was used to solubilize the formazan dye. The colour of the solution was determined by

spectrophotometer using DMSO as a blank. Finally, the difference in cell death was calculated as a relative percentage of cell viability. The released LDH activity was determined from the collected media using the LDH activity assay kit.

2.13. Statistical analysis

In this study, statistical analysis was calculated by using commercially available software (GraphPad Prism version 8, San Diego, CA, USA). All data were expressed as mean ± SEM. All comparisons were determined a significance using an unpaired t-test or ANOVA, followed appropriately by the Tukey–Kramer test. A p-value of less than 0.05 was used to indicate statistical significance.

CHAPTER 4

Results and Discussion

3.1. Physical characteristics of nanoparticles

The FE-SEM was used to identify and determine the morphology of nanoparticles. The results showed that SNPs had a round shape with a smooth surface (Figure 24a, d), whereas GSNPs and rhSLPI showed a round shape with a rough surface (Figure 24b, c, e, f). Each type of nanoparticle has similar diameters, which were measured by ImageJ. Sizes of SNPs, GSNPs, and rhSLPI-GSNPs are 273.3 ± 6.33 nm, 300.3 ± 6.55 nm, and 301.3 ± 12.73 nm, respectively (Figure 25a). The size of GSNPs and rhSLPI-GSNPs were larger than the size of SNPs ($p < 0.05$). PDI and zeta potential values of nanoparticles that were obtained using Zetasizer. The PDI value of SNPs, GSNPs, and rhSLPI-GSNPs are 0.05 ± 0.02, 0.47 ± 0.10, and 0.51 ± 0.10, respectively (Figure 25b). The PDI of GSNPs and rhSLPI-GSNPs was significantly higher than the PDI of SNPs ($p < 0.05$). The surface of nanoparticles has an impact on the zeta potential values. SNPs, GSNPs, and rhSLPI-GSNPs have zeta potentials value of -57.27 ± 1.00 mV, -22.40 ± 1.37 mV, and -24.50 ± 1.92 mV, respectively (Figure 25c). The zeta potential of GSNPs and rhSLPI-GSNPs was significantly higher than the zeta potential of SNPs ($p < 0.05$).

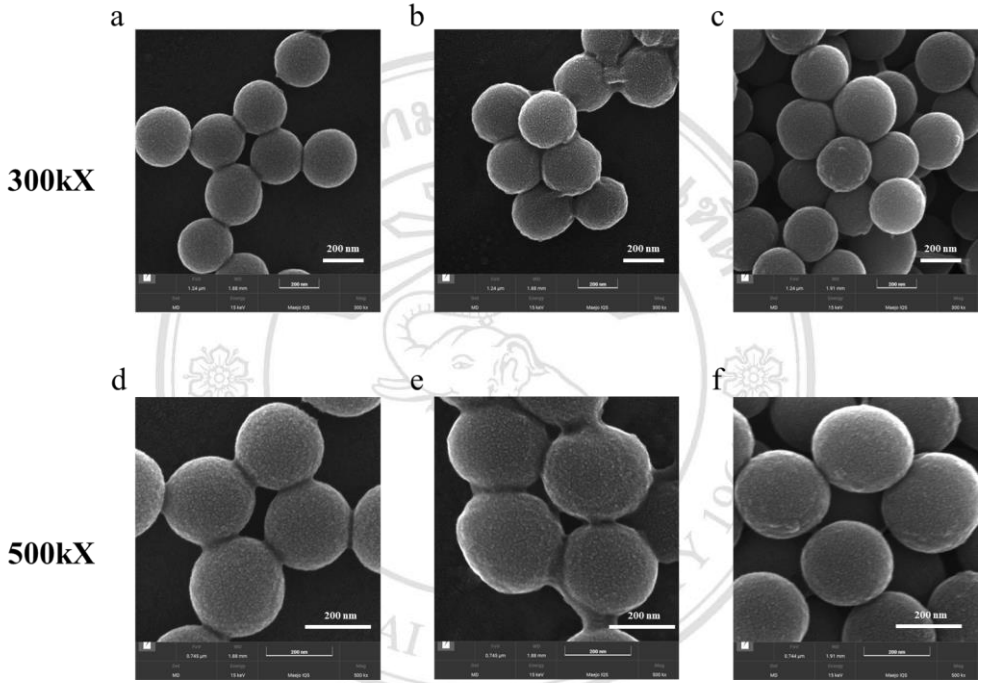


Figure 24 Morphology of nanoparticles. a) SNPs (300kX). b) GSNPs (300kX). c) rhSLPI-GSNPs (300kX). d) SNPs (500kX). e) GSNPs (500kX). f) rhSLPI-GSNPs (500kX).

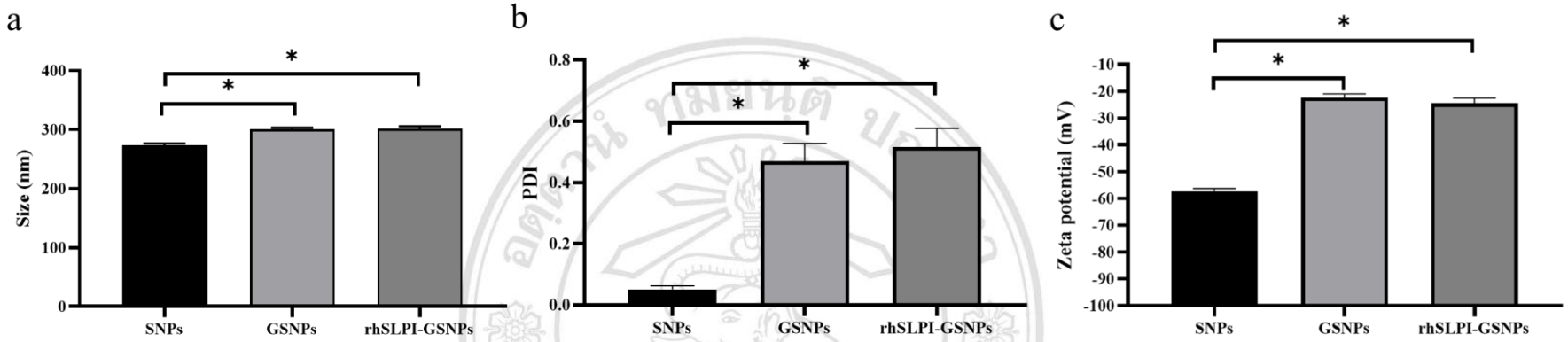


Figure 25 Characterizations of nanoparticles. a) size. b) PDI. c) zeta potential. * $p < 0.05$ versus each other group (ANOVA)

Previous studies reported a cardioprotective effect of SLPI in decreased cell injury *in vitro* studies [12], and decreased infarct size in the heart of rats in an *ex vivo* study [41]. However, SLPI has a short half-life *in vivo* due to protease enzymes in circulation [17]. Therefore, a delivery system is necessary, and this study attempted to prepare a peptide delivery by using GSNPs. The present study demonstrated for the first time that synthesized GSNPs encapsulating rhSLPI could provide a cardioprotective effect against an *in vitro* simulated ischaemia/reperfusion injury in the cardiac cell.

In the study, the surface of synthesized SNPs initially showed a smooth surface (Figure 24a), and both surfaces of synthesized GSNPs and rhSLPI-GSNPs were later changed to a rough surface after coating with a thin layer of gelatine (Figure 24b). Typically, the surface of SNPs is quite smooth surface, however when covered with gelatine increased rough surface was observed due to the formation of gelatine on the surface of SNPs. Gelatine could be adsorbed on the surface of SNPs, therefore gelatine was cross-linked by glutaraldehyde, which carboxaldehyde (CHO) of glutaraldehyde interacts with the amine (NH_2) of gelatine [157] for preventing the adsorbed gelatine dissolution [145].

The size of GSNPs and rhSLPI is larger than SNPs showing that covered gelatine increased the diameter by approximately 30 nm indicating that the layer of covered gelatine is 15 nm. The PDI value of particles depends on the size and distribution of nanoparticles, varying sizes of nanoparticles could provide a high PDI value compared to an equivalent size. The suitable PDI value is 0.01 to 0.7, which indicated monodispersed particles, whereas the PDI value > 0.7 indicated the broad particle size distribution of the formulation [158]. The result of SNPs nanoparticles showed that a similar size that made a PDI 0.05 ± 0.02 (Figure 25b) is monodispersed particles. Both of GSNPs and rhSLPI-GSNPs showed a value of PDI in 0.1 to 0.7 indicating that nanoparticles are monodispersed particles. However, PDI increased as a result of the aggregation of particles.

The electric charge on the nanoparticle's surface was measured by zeta potential in which the charge of SNPs became more positive after coating with gelatine (GSNPs) and loading rhSLPI (rhSLPI-GSNPs). The surface charge of SNPs showed negatively charged from the silanol groups [159], while GSNPs and rhSLPI-GSNPs were slightly increased due to positively charged gelatine coating [160], indicating a successful coating process. The stability of particles depends on the zeta potential value at a specific surface potential, usually ± 35 mV, particles tend to prevent coagulation via electrostatic repulsion [161]. As explained following Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, when electrostatic repulses, driven by similarly charged electrical layers surrounding the particles, triumph over the attraction of the Van Der Waals interactions, particles become dispersible [161, 162]. The zeta potential of GSNPs and rhSLPI-GSNPs is lower than + 35 mV and higher than -35 mV, resulting, in particles occurring the aggregation, which had made increased the PDI value. SLPI, which is coated on the surface of NPs without a gelatine coating, is not effectively protected when delivered in circulation since protease enzymes are likely to destroy it. Another layer can be shielded from destruction by being covered in gelatine.

3.2. Determination of SLPI protein contained in rhSLPI-GSNPs

The results showed that GSNPs did not find any SLPI compared to 1 mg of rhSLPI-GSNPs gave SLPI at 235 ng (Figure 26). rhSLPI-GSNPs had a significantly SLPI concentration higher than GSNPs ($p < 0.05$). As a result, the calculated mass of SLPI from 50 mg of synthesized rhSLPI-GSNPs, which is the mass used for fabrication, was approximately 11.75 µg. The mass of rhSLPI, which loaded in nanoparticles was calculated by comparing the default value of rhSLPI that was 165 µg for calculating encapsulation efficiency (EE %) as 7.12%.

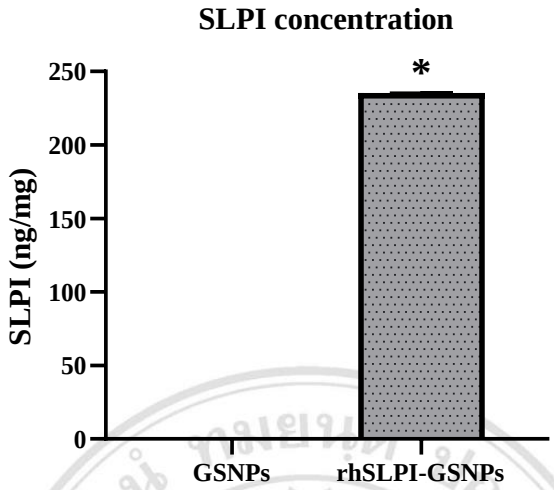


Figure 26 Determination of SLPI concentration from synthesized nanoparticles GSNPs and rhSLPI-GSNPs. * $p < 0.05$ (Unpaired t-test)

In this study, we successfully encapsulated the rhSLPI, which can be seen in Figure 26 which shows that rhSLPI-loaded nanoparticles (GSNPs) contain SLPI protein. Considering the concentration of rhSLPI used, the amount of rhSLPI loaded into the NPs was quite minimal, potentially due to the volume of rhSLPI depends on the surface of the nanoparticle because rhSLPI can only bind to the surface of the core SNP that may be less loaded than the coreless or smaller nanoparticles. GSNPs can encapsulate rhSLPI due to rhSLPI having a cationic charge (+ 12), which consists of an N-terminal (+7) and a C-terminal (+5) [14]. As a result, the cationic charge of rhSLPI likely attaches to an anionic charge surface of SNPs when the breakdown of the gelatine coating rhSLPI is released and measured.

Other perspective reasons, gelatine and rhSLPI compete to bind the surface of SNPs. The rhSLPI may be separated from SNPs by centrifugation method, resulting gelatine could attach on some space of surface replace rhSLPI.

Although GSNPs can encapsulate SLPI, they can encapsulate SLPI to 7.12%, when compared to other previous studies of silica nanoparticles. Mesoporous silica nanoparticles (MSN) were used to encapsulate bovine serum albumin (BSA), they had a size of 168 ± 10 nm and a percentage of encapsulation efficiency of 33% [163]. Porous silica nanoparticles (pSiNP) had a size of 130 nm for encapsulating brain-derived neurotrophic factor (BDNF), and the percentage of encapsulation efficiency as 13% [164]. Both of these types have a hole for the protein to be interred. However, in this study, GSNPs did not have a pore to provide more surface area for peptide capturing. As a result, GSNPs can encapsulate little SLPI.

3.3. Drug releasing

The drug release study was separated into 2 protocols including performing the releasing profile in PBS solution up to 72 h, and performing the releasing profile in 0.1% collagenase solution up to 240 min. Figure 27a shows the releasing-profile of rhSLPI from rhSLPI-GSNPs in PBS solution at 0 h, 24 h, 48 h, and 72 h. The accumulative percentage of release was 0%, 0.26%, 0.97%, and 1.05%, respectively. The results showed that rhSLPI could be released less than 2% in 72 h.

Different study protocol was performed the releasing profile in 0.1% collagenase solution up to 240 min. The result showed the accumulative release of rhSLPI from rhSLPI-GSNPs, in 0.1% collagenase solution for 0 min, 15 min, 30 min, 60 min, 120 min, and 240 min, was 0%, 13.97%, 30.96%, 52.28%, 74.73%, and 100%, respectively. The results showed that rhSLPI could be released 100% within 240 min (Figure 27b).

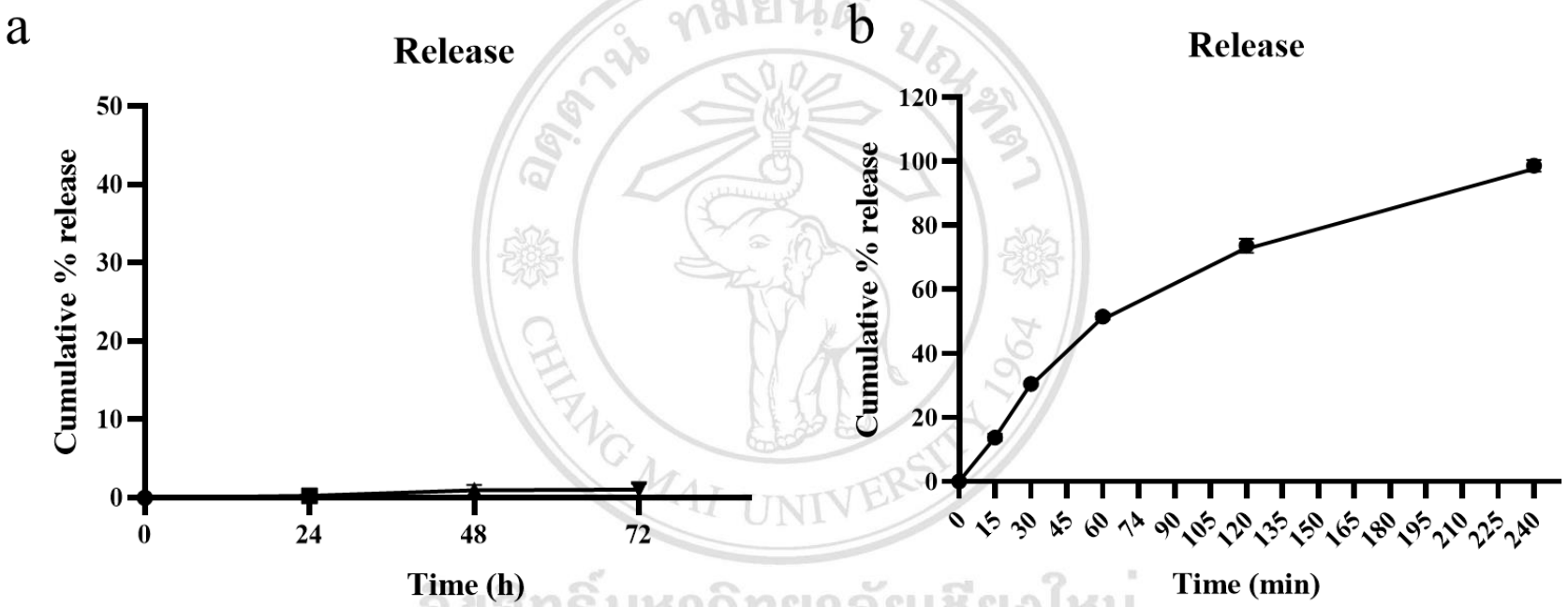


Figure 27 Release profiles of rhSLPI from rhSLPI-GSNPs. a) In PBS solution. b) In 0.1% collagenase solution.

The releases of rhSLPI in PBS solution are 1% from the beginning to the 72 hrs due to the thick layer of gelatine, which covered the SNPs, could not be degraded in PBS due to the cross-linked process by glutaraldehyde (Figure 27a). The result of rhSLPI release in PBS is similar to previous studies, which determined gelatine cover mesoporous silica nanoparticles (GMSNs). The GMSNs can release drug with different pH concentrations (pH 7.4 is rarely released, and low pH is more released), indicating that covered gelatine is pH-dependent degrading [145]. However, low pH decreased the activity of SLPI. Thus, this releasing protocol seems unpractical in this study.

We perform the alternative strategy for drug releasing assay by using enzymatic digestion to assist degradation of gelatine, which could easily release the rhSLPI to the solution. In a previous study, when gelatine is broken down, protein is released, but how quickly this happens depends on the enzyme's concentration. Gelatinase A was employed in earlier investigations to degrade the gelatine layer [146], which is similar to our findings. Another enzyme that can degrade gelatine is collagenase. The result showed that collagenase can provide rhSLPI release from GSNPs in 240 min (Figure 27b). Gelatine can be digested by a large number of enzymes found in living plasma, making additional research into the *in vivo* release of gelatine important.

The glutaraldehyde can crosslink a C-terminal region of protein such as gelatine. In this study, not only gelatine could be cross-linked, but also rhSLPI. Therefore, enzymatic digestion of gelatine for drug releasing possibly pulled rhSLPI together with the gelatine. rhSLPI in crosslinking form might not be able to be recognized by the SLPI antibody, and therefore the detection of SLPI by ELISA was low, and hence cause low percentage of calculated encapsulation efficiency.

3.4. Cardiac cytotoxicity of NPs

Concentrations of GSNPs and rhSLPI-GSNPs were varied by 10-fold dilution from 0 to 100 μg/mL and incubated in cardiomyoblast (H9c2) cells for 24 h (Figure 28). The results showed no significant difference in cell viability of cells treated with GSNPs or rhSLPI-GSNPs in every concentration ($p < 0.05$). There are two concentrations of rhSLPI-GSNPs including 1 μg/mL, and 100 μg/mL, which had a percentage of cell viability below 95% (Lethal dose 5, LD5) (92.11 ± 10.27%, and 84.80 ± 2.47%, respectively).

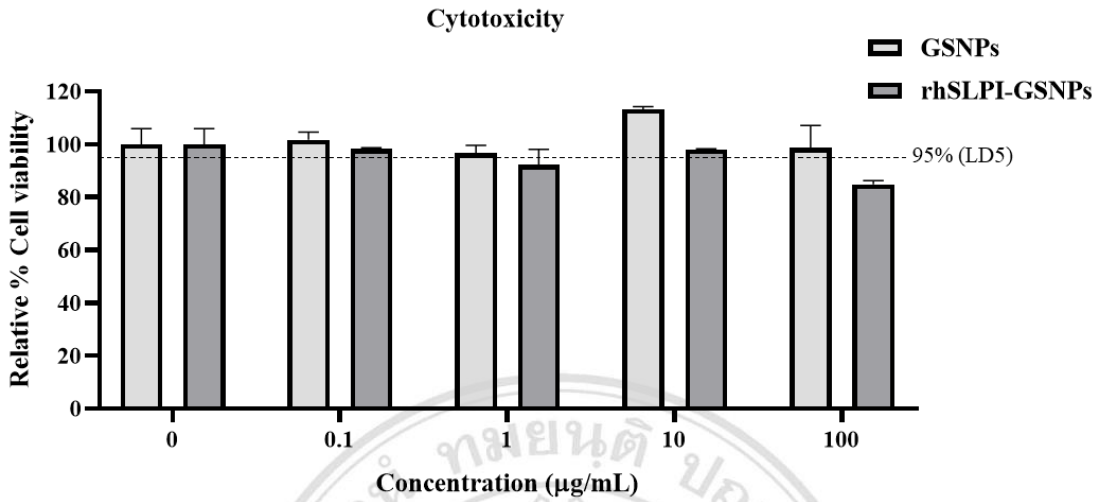


Figure 28 The cytotoxicity test by determining the relative percentage of cell viability among nanoparticles treated groups (0.1, 1, 10, 100 µg/ml) vs. untreated control group. * $p < 0.05$ (ANOVA).

To ensure that the nanoparticles generated from this study could provide a therapeutic effect without harming the cells which could cause adverse effects when used in real clinical treatment, the cytotoxicity of the cardiac cell must be determined. This study demonstrates the cytotoxicity of GSNPs within and without rhSLPI. Both GSNPs and rhSLPI-GSNPs were found to be non-toxic to cardiac cells. This could be because silicon dioxide is biocompatible, which has been used in nanomedicine and is safe, but a previous study showed that silicon nanoparticle toxicity is determined by its size and dosage [165]. Gelatine is a protein found in bones, tendons, ligaments, and skin, and rhSLPI is a protein found in human secretion [11]. Therefore, both gelatine and SLPI are considered friendly molecules for the human body which suggests that both molecules could be used for treatment in humans.

Neither concentration was toxic to cardiac cells. However, rhSLPI-GSNPs at concentrations of 1 ug/mL and 100 ug/mL had less than 95% survival (LD5). The result suggested that 1 ug/mL may be a concentration that is slightly toxic to cells, but there was not statistically significant difference. However, rhSLPI-GSNPs at 10 ug/mL may cause greater toxicity and cause cell death. But there may be a sufficient effect of SLPI on cell survival. At 100 ug/mL, the concentration may be greater toxic to the cells.

3.5. Optimization of sI/R

Optimization of sI/R time was managed from 0 to 60 mins and measured by MTT assay and released LDH assay. The result showed the percentage of cell viability of time 0 min to 60 min was 100.00 ± 19.55%, 91.77 ± 13.34%, 88.07 ± 13.85%, 83.14 ± 11.75%, 75.02 ± 9.23%, 65.21 ± 11.98%, and 55.76 ± 6.27%, respectively (Figure 29a). In a time of 40 min, 50 min, and 60 min cell viability was significantly lower than the control group (0 min) ($\rho < 0.05$). The result showed that the cell viability was reduced in a time-dependent manner. The results also showed that the duration of sI/R that cause 50% of cell death [166] and cell injury was 60 min (Figure 29a) with cell viability at 55.76 ± 6.27%. Therefore, 60 min ischaemia with 24 h reperfusion was the optimum treatment condition that was used for further studies for determining the sI/R injury-induced cell death with rhSLPI-GSNPs. The result of the released LDH of time 0 min to 60 min was 0.45 ± 0.03, 0.47 ± 0.06, 0.52 ± 0.04, 0.58 ± 0.05, 0.71 ± 0.05, 0.74 ± 0.07, and 1.02 ± 0.12, respectively (Figure 29b). In a time of 40 min, 50 min, and 60 min cell viability was significantly higher than the control group (0 min) ($p < 0.05$).

Optimization of ischaemic time for the sI/R injury model was the sI/R at 60 min significantly reduced cell viability by approximately 50% (Figure 29a). The viability of the cell was deceased below 95% after 10 min, however, the result showed a significantly decreased at 40 min similar to released LDH activity (Figure 29b).

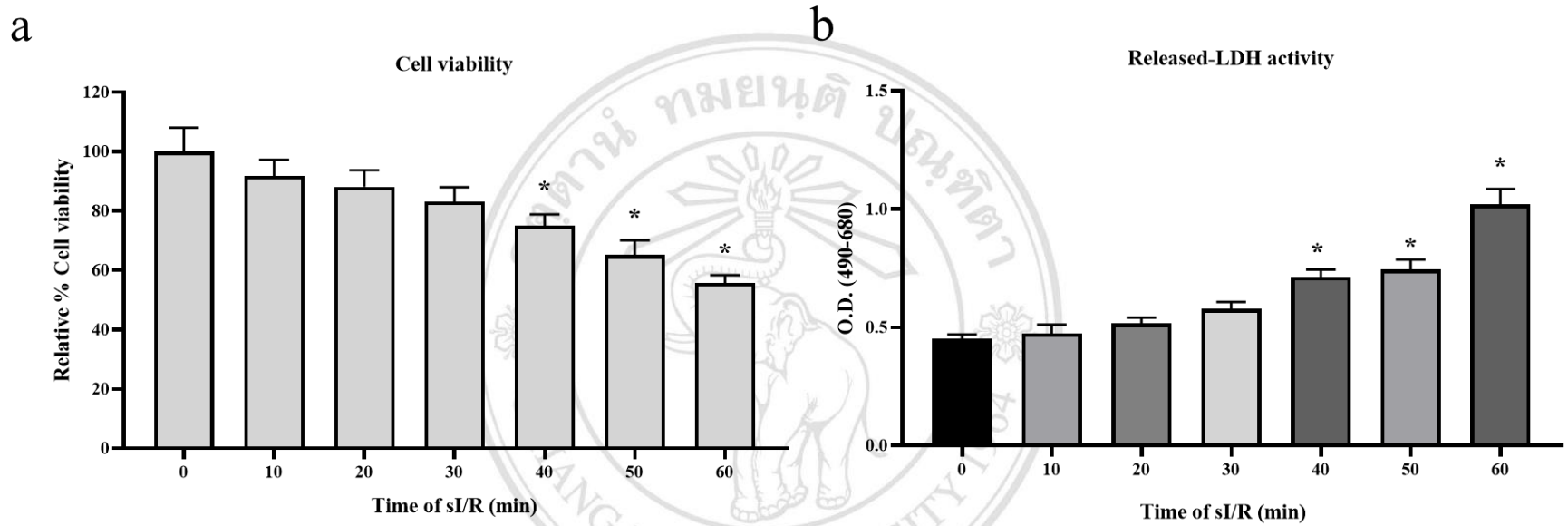


Figure 29 Optimization of ischaemic time for sI/R injury model. a) determined cell viability by MTT viability assay; b) determined cell injury by released LDH activity. * $p < 0.05$ vs untreated control (ANOVA).

3.6. Cardioprotective effect of rhSLPI-GSNPs against an *in vitro* sI/R in cardiac cells

The result showed that sI/R could significantly reduce the percentage of cell viability when compared to control group (54.72 ± 12.78% vs 100.7 ± 13.34%, $p < 0.05$). Treatment of rhSLPI-GSNPs at 1, 10, and 100 μg/mL significantly improved the percentage of cell viability ($p < 0.05$) when compared to the sI/R group (55.22 ± 13.26%, 72.44 ± 9.83%, and 56.01 ± 16.75%, respectively vs 54.72 ± 12.78%, $p < 0.05$) (Figure 30a). The sI/R increased the released LDH activity when compared to control group (0.77 ± 0.06 vs 0.34 ± 0.03). Treatment with rhSLPI-GSNPs at concentration 1, 10, and 100 μg/mL could significantly reduce released LDH activity (0.68 ± 0.06, 0.69 ± 0.06, and 0.68 ± 0.04, $p < 0.05$ respectively) (Figure 30b).

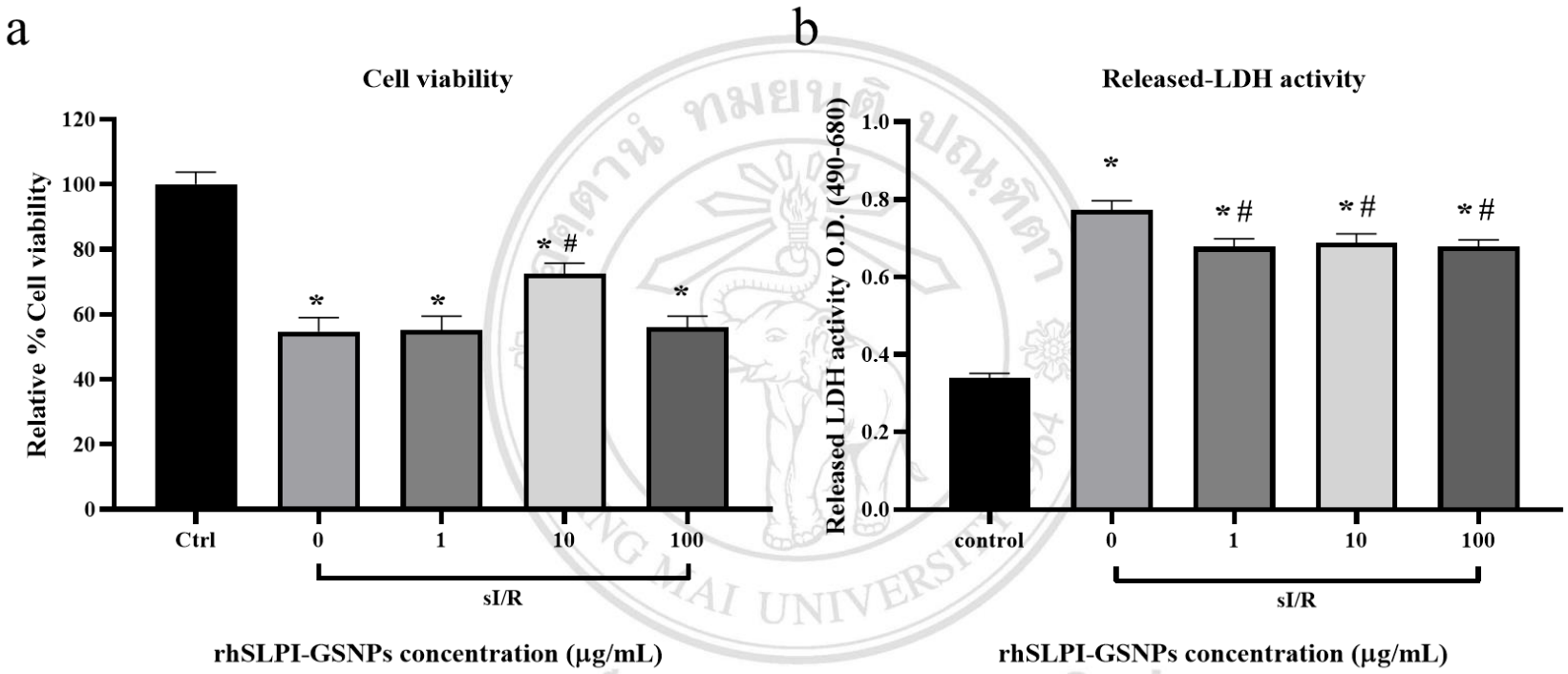


Figure 30 An *in vitro* cardiac protective effect of rhSLPI-GSNPs against sI/R in cardiac cells. a) determined cell viability by MTT viability assay; b) determined cell injury by released LDH activity. * $p < 0.05$ versus control group (ANOVA), # $p < 0.05$ versus sI/R groups (ANOVA).

The cardioprotective effect of rhSLPI has been demonstrated in an *in vitro* simulated I/R injury in cardiac cells. A previous study showed that the effective concentration of rhSLPI that could provide an *in vitro* cardioprotection was 1,000 ng/mL [166]. However, in this study, treatment of 1, 10, and 100 μg/mL of nanoparticles suggest treatment of rhSLPI at concentrations of 0.23, 2.35, and 23.5 ng/mL, respectively (Figure 30a). This amount of rhSLPI from NPs was found to be very different from the concentration of rhSLPI used in previous studies. The current study showed that 10 μg /mL of rhSLPI-GSNPs, which contain a rhSLPI concentration of 2.35 ng/mL (Figure 30a), could sufficiently protect against cardiac cell injury and death in an *in vitro* sI/R injury. This concentration is much lower than the concentration of rhSLPI used in previous studies of 1,000 ng/mL [166].

As a result of cytotoxicity, concentrations at 1, and 100 μg/mL of rhSLPI-GSNPs showed cell viability lower than 95%, which indicated that rhSLPI-GSNPs can cause cell death. However, as the concentration at 1 μg/mL encapsulated too little rhSLPI, as a result, SLPI is the unable to protect the cell from sI/R. The 100 μg/mL of rhSLPI-GSNPs is a bad concentration that causes cell death because of their toxicity. Although there is a high SLPI concentration, it does not reduce cell death from nanoparticle's toxicity.

From the results, it appears that 10 μg /mL of rhSLPI-GSNPs was the most effective dose to protect the cardiac cell from sI/R injury. Increasing the concentration of rhSLPI-GSNPs to 100 μg/mL might not top up the protective effect. However, it could be due to the beneficial effect of rhSLPI to inhibit the generation of intracellular ROS, attenuation of p38 MAPK activation, and activate pro-survival kinase- Akt, as well as reduced the expression levels of apoptotic proteins [166].

The result of released LDH activity has differed to cell viability which is probably because mitochondria still play a role to synthesize mitochondrial reductase, which transforms MTT to formazan by MTT assay, however, the cell may be injured and produce LDH from sI/R and release pass cellular membrane of the cell, as a result, it can be detected by LDH activity (Figure 30b).

The rhSLPI-GSNPs of this study could be potentially used in several applications for improving strategical treatment. Percutaneous coronary intervention (PCI) is one of revascularization techniques used to open clogged coronary arteries by using stent, which help keep the artery from narrowing or closing of blood. Coating rhSLPI-GSNPs on the surface of a stent could be slightly released rhSLPI from nanoparticles to reduce disease severity of cardiovascular diseases and improve cardiac function. In addition, rhSLPI-GSNPs could be preservative agent of blood vessels to prevent damage during transportation. We hope that this nanoparticle could be alternative therapeutic agent for treatment of IHD and other cardiovascular diseases.

CHAPTER 5

Conclusion

SLPI loaded nanoparticles in previous studies have reported to encapsulating in three types that are liposome, alginate/chitosan, and human serum albumin and have not reported in other nanoparticles. This is the first study to synthesize gelatine-covered silica nanoparticles (GSNPs) to encapsulate rhSLPI (rhSLPI-GSNPs). We found the rhSLPI-GSNPs provides cardioprotective effect, which against an *in vitro* simulated ischaemia/reperfusion injury in cardiac cells. This study provides a useful information for rhSLPI-GSNPs in the cardioprotective effect. However, this method is required more investigated in the ischaemic heart disease treatment in term of a pre-clinical animal models.

Limitations of study and suggestions

There are several points that could be addressed as the limitations of the current study.

We did not perform gelatine layer assessment by a transmission electron microscope (TEM). In previous studies on gelatine-coated silica nanoparticles, the surface differences between bare and gelatine-coated nanoparticles were reported because a scanning electron microscope (SEM) could not identify between the surfaces of each nanoparticle. In order to conduct further research, I recommend that determination of gelatine layer by using transmission electron microscope (TEM) for identify the surfaces between each nanoparticle.

In this study, intracellular uptake was not performed due to lack of fluorescein-labelled peptides. A previous report on intracellular uptake of SLPI, the SLPI protein is required to be conjugated with fluorescein, subsequently purified the labelled-SLPI, which then used for determining intracellular uptake [122]. Future study, cellular uptake has to determine by labelling SLPI by using fluorescein before encapsulating into nanoparticles. determine cellular uptake by using a fluorescence microscope. Another technique as A confocal laser scanning microscope is a one technique to determine the intracellular uptake of nanoparticles, it use to resolve the detailed structure of objects such as nanoparticles within the cell. However, is not have machine in Chiang Mai.

In this study, MTT cell viability assay was used to determine cell viability. The principle of the method is measuring the mitochondrial oxidoreductase activity and indirectly reflect the viability of the cells. However, some stress could alter intracellular oxidative state and could also provide unreliable results. The cell viability should be determined by using trypan blue stain, which is gold standard technique [167]. Cell viability has to measure by using gold standard as trypan blue stain assay, which is higher sensitivity than MTT assay.

In this study, we attempt to determine the anti-protease activity of SLPI from rhSLPI-GSNPs, for ensuring that SLPI encapsulated in nanoparticles can still work. However, breaking of rhSLPI-GSNPs in this study seems unfavourable for determining anti-protease activity of SLPI. We tried to determine the effect of rhSLPI-GSNPs for inhibit the neutrophil elastase activity, which is the well-known enzyme that could potentially be inhibited by SLPI. In addition, we also determine the anti-trypsin activity of rhSLPI-GSNPs by using casein hydrolysis in both colorimetric activity assay (Figure 31a) and casein zymography (Figure 31b). However, it seems that the results could not provide conclusive data. The explanation is due to using of collagenase as the accelerator of SLPI release (as seem in result figure 18b), leftover collagenase enzyme in sample could interfere the assessment of anti-protease activity of SLPI that release from rhSLPI-GSNPs. Moreover, breaking gelatine from rhSLPI-GSNPs for release rhSLPI, by using other techniques such as heat and pH, could also interfere the activity of rhSLPI, due to protein denaturation.

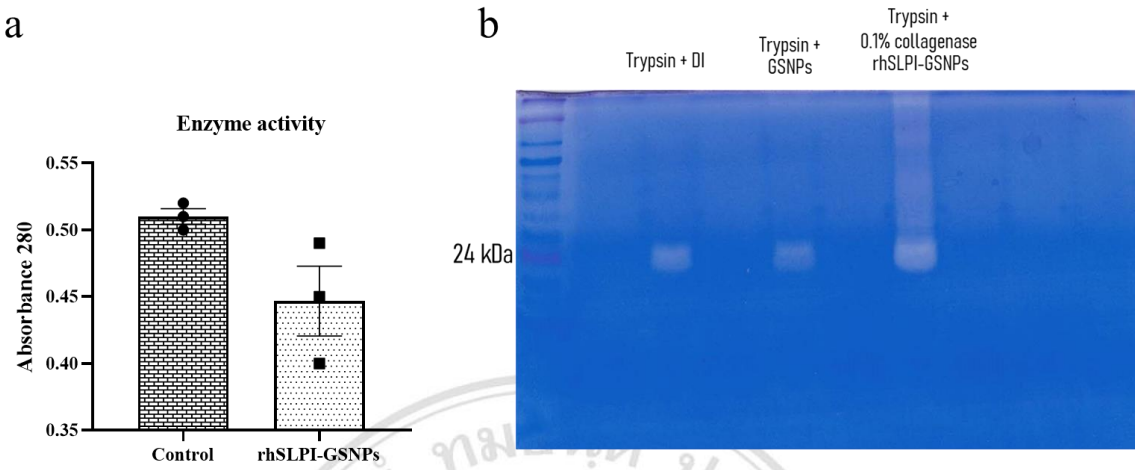


Figure 31 Enzymatic activity of SLPI loaded in nanoparticles. a) trypsin inhibiting; b) casein zymography

Future prospective of the study

The treatment of rhSLPI-GSNPs in the H9c2 cell is not directly connected to actual physiological conditions in the intact heart due to it do not exhibit the electrophysiological property. Therefore, a treatment of rhSLPI-GSNPs in real models such as in intact heart may provide more useful information similar to real physiological events in the heart. An experiment in real model such as *ex vivo* and *in vivo* models might show efficiency of cardioprotective effect of rhSLPI-GSNPs against I/R injury such as reduction of infarct size, decreasing of ROS expression, reduction of inflammatory cytokines, as well as cardiac biomarker such as cardiac troponin I, CK-MB, and myoglobin and improve cardiac function which is providing the therapeutic potential of rhSLPI. Thus, the more physiological cardiac models in an *ex vivo* and *in vivo* study of I/R injury, need to be intensively investigated, which could provide functional data for further clinical trials for being therapeutic treatment.

The optimization of nanoparticles is required. A size of particles affects to an encapsulation of peptide loading, small size gives more surface than large size. However, too small size can induce ROS generation and induce cell death. In further study, variety of nanoparticle's size could provide useful information and more efficiency of encapsulation. A crosslinking of gelatine by glutaraldehyde might affect to half-life of rhSLPI-GSNPs in a body. An optimization of glutaraldehyde concentration is required to determine the degradation of nanoparticles in animal models.

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CURRICULUM VITAE

Author's Name	Mr.Faprathan Pikwong
Place of Birth	Lamphun, Thailand
Education	2016 High School Certificate from Ban Hong Rattana Wittaya School, Lamphun
	2020 Bachelor of Science Program in Medical Technology from Naresuan University, Phitsanulok
Scholarship	TA&RA Scholarship 2021 from Graduate school, Chiang Mai University, Chiang Mai
	Biomedical Engineering Institute (BMEI), Chiang Mai University and ACADEMIC ACTIVITIES SCHOLARSHIP- Graduate school, Chiang Mai University for travel grant to attend International Conference and Exhibition on Science, Technology and Engineering of Materials (ISTEM2022). November 29-December 3, 2022. Pattaya, Cholburi, Thailand.
Publications	1. **Pikwong F**., Chouyratchakarn W., Phutiyothin C., Chanabodeehalermrung B., Baipaywad P., Kumphune S. "Development of gelatin-silicon dioxide nanoparticles (GSNPs) encapsulating recombinant human secretory leukocyte protease inhibitor (rhSLPI) for reducing *in vitro* myocardial ischaemia/reperfusion injury," The International Conference and Exhibition on Science, Technology and Engineering of Materials (ISTEM 2022), 2022

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3. **Pikwong F.**, Phutiyothin C., Chouyratchakarn W., Baipaywad P., Kumphune S. Gelatin coated -Silicon oxide nanoparticles encapsulated recombinant human secretory leukocyte protease inhibitor (rhSLPI) reduced cardiac cell death against an in vitro simulated ischaemia/reperfusion injury. *Materials Today: Proceedings,* 2023 *(Accepted)*

4. Mongkolpathumrat P., Chouyratchakarn W., **Pikwong F.**, Phutiyothin C., Kumphune S. Roles of secretory leukocyte protease inhibitor (SLPI) in non-communicable diseases and study models. 2023 *(Manuscript in preparation)*

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