DEVELPOMENT OF SILYMARIN-LOADED POLYMERIC MICELLES FOR PREVENTION OF CISPLATIN-INDUCED NEPHROTOXICITY

By

Miss Wajee Tipparos

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

Master of Pharmacy Program in Pharmaceutical Sciences

Graduate School, Silpakorn University

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การพัฒนาพอลิเมอริกไมเซลล์บรรจุซิลิมารินสำหรับป้องกันความเป็นพิษต่อไตจากยาซิสพลาทิน

โดย
นางสาววจี ทิพรส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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The Graduate School, Silpakorn University has approved and accredited the thesis title of “Development of Silymarin-loaded Polymeric Micelles for Prevention of Cisplatin-induced nephrotoxicity” submitted by Miss Wajee Tipparos as a partial fulfillment of the requirements for the degree of Master of Pharmacy in Pharmaceutical Sciences.

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WAJEE TIPPAROS: DEVELPOMENT OF SILYMARIN-LOADED POLYMERIC MICELLES FOR PREVENTION OF CISPLATIN-INDUCED NEPHROTOXICITY. THESIS ADVISORS: ASSOC. PROF. PRANEET OPANASOPIT, Ph.D. AND ASSOC. PROF. THEERASAK ROJANARATA, Ph.D. 103 PP.

Nephrotoxicity is a major side effect which limits the use of cisplatin. Nevertheless, silymarin (SM) has been previously reported to have renoprotective effect against this drug. Since SM is poorly water soluble, this study aimed to incorporate SM into the polymeric micelles made from amphiphilic chitosan derivatives; (*N*-benzyl-*N*,*O*-succinyl chitosan (BSCS);*N*-octyl-*N*-*O*-succinyl chitosan (OSCS);*N*-naphthyl-*N*,*O*-succinyl chitosan (NSCS) using various physical entrapment methods to increase the solubility. SM-loaded BSCS, NSCS and OSCS micelles were successfully prepared via both evaporation and sonication method as well as cosolvent evaporation method with high entrapment efficiency and loading capacity. SM-loaded OSCS micelles showed the highest entrapment efficiency and loading capacity. An increase of the initial amount of silymarin resulted in an increasing in particle size. The particle size was in the range of 160 – 336 nm. All silymarin-loaded micelles were negatively charged. The release behavior of SM-loaded polymeric micelles in gastrointestinal tract (pH 1.2, then changed to 6.8) and phosphate buffer saline pH 7.4 medium was significantly higher than free SM. The *in vitro* permeation rate of SM-BSCS and SM-OSCS were faster than free drug due to the enhanced solubility. The cytotoxicity study showed that free SM and SM-loaded polymeric micelles had a low toxicity against renal cells (RPTEC/TERT1) and could kill head and neck cancer cells (HN22) viability at the high dose. Pre- or co-treatment of free SM and SM-loaded polymeric micelles did not reduce the killing effects during the treatment with cisplatin, as evaluated by MTT assay. Free SM and SM-loaded polymeric micelles pre-treatment at the concentration of 50 and 100 µg/mL resulted in the renoprotective effects against cisplatin-induced nephrotoxicity. The % cell viability of free SM and SM-loaded polymeric micelles pre-treatment group after cisplatin treatment was higher than that without pre-treatment. Additionally, cell apoptosis and necrosis in the RPTEC/TERT1 cells pre-treatment with SM-loaded polymeric micelles was reduced, compared with cisplatin treatment group. It is suggested that incorporation of SM into polymeric micelles can increase its solubility and exhibit potential nephroprotective effects against cisplatin toxicity.

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คำสำคัญ : ซิลิมาริน/พอลิเมอริกไมเซลล์/ความเป็นพิษต่อไตที่เกิดจากยาซิสพลาทิน/อนุพันธ์ของไคโตซาน

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ความเป็นพิษต่อไตเป็นอาการข้างเคียงที่จำกัดการใช้ยาซิสพลาทิน อย่างไรก็ตามมีรายงานว่าซิลิมารินมีฤทธิ์ในการปกป้องไตจากยาซิสพลาทิน เนื่องจากซิลิมารินเป็นสารที่มีค่าการละลายน้ำต่ำนั้น ดังนั้นในการวิจัยนี้จึงมีวัตถุประสงค์เพื่อพัฒนาพอลิเมอริกไมเซลล์จากอนุพันธ์ของไคโตซาน(*N*-benzyl-*N,O*-succinyl chitosan (BSCS); *N*-naphthyl-*N,O*-succinyl chitosan (NSCS); *N*-octyl-*N*-*O*-succinyl chitosan (OSCS) สำหรับบรรจุและเพิ่มการละลายของยาซิลิมารินด้วยวิธีทางกายภาพ โดยพบว่าสามารถบรรจุซิลิมารินในพอลิเมอริกไมเซลล์ BSCS, NSCS และ OSCS ด้วยวิธีระเหยแห้งร่วมกับคลื่นความถี่สูง และวิธีระเหยตัวทำละลายร่วม โดยพบว่าซิลิมารินที่ถูกบรรจุใน OSCS มีประสิทธิภาพในการบรรจุยาได้มากที่สุด เมื่อเพิ่มปริมาณตัวยาเริ่มต้นส่งผลให้ไมเซลล์มีขนาดอนุภาคใหญ่ขึ้น โดยมีขนาดอนุภาคอยู่ในช่วง 160 – 336 นาโนเมตร และแสดงประจุลบ การปลดปล่อยยาจากซิลิมารินพอลิเมอริกไมเซลล์ในสารละลายที่จำลองสภาวะระบบทางเดินอาหาร (กระเพาะอาหาร พีเอช 1.2 และเปลี่ยนเป็นลำไส้ พีเอช 6.8) และพีบีเอส พีเอช 7.4 พบว่าการปลดปล่อยซิลิมารินจากซิลิมารินที่บรรจุในพอลิเมอริกไมเซลล์นั้นมีค่าสูงกว่าซิลิมารินเปล่า รวมถึงการศึกษาการดูดซึมผ่านผนังลำไส้ พบว่าการดูดซึมผ่านของซิลิมารินที่บรรจุในพอลิเมอริกไมเซลล์ BSCS และ OSCS นั้นมีค่าสูงกว่าซิลิมารินที่ซิลิมารินเปล่า เนื่องจากพอลิเมอริกไมเซลล์สามารถช่วยเพิ่มการละลายของยาซิลิมารินได้ จากการศึกษาความเป็นพิษต่อเซลล์เพาะเลี้ยง พบว่าซิลิมารินมีความเป็นพิษต่ำต่อเซลล์ไต (RPTEC/TERT1) และเมื่อใช้ในปริมาณสูงจะสามารถลดการเจริญของเซลล์มะเร็งหัวและคอ (HN22) ได้ การให้ซิลิมารินเปล่าและซิลิมารินบรรจุพอลิเมอริกไมเซลล์ร่วมกับการใช้ยาซิสพลาทินทั้งก่อนและให้ร่วมกัน พบว่าซิลิมารินและซิลิมารินบรรจุพอลิเมอริกไมเซลล์ไม่มีผลในการลดประสิทธิภาพของยาซิสพลาทินต่อเซลล์มะเร็ง การให้ซิลิมารินและซิลิมารินบรรจุพอลิเมอริกไมเซลล์ที่ความเข้มข้น 50 และ 100 ไมโครกรัมต่อมิลลิลิตร ก่อนการให้ยาซิสพลาทิน พบว่าซิลิมารินและซิลิมารินบรรจุพอลิเมอริกไมเซลล์มีฤทธิ์ในการปกป้องไตจากความเป็นพิษของยาซิสพลาทินได้ โดยร้อยละของเซลล์ที่อยู่รอดมีค่าสูงกว่าเซลล์ที่ไม่ได้รับซิลิมารินและซิลิมารินบรรจุพอลิเมอริกไมเซลล์ นอกจากนี้การตรวจสอบการตายของเซลล์ยังพบว่าการให้ซิลิมารินและซิลิมารินบรรจุพอลิเมอริกไมเซลล์ก่อนการให้ซิสพลาทินสามารถลดการตายของเซลล์ไตแบบอพ็อพโทรซิสและเนโครซิสได้ สรุปได้ว่าการบรรจุซิลิมารินลงในพอลิเมอริกไมเซลล์สามารถช่วยเพิ่มการละลายของยาได้ และยังมีฤทธิ์ในการปกป้องความเป็นพิษต่อไตจากยาซิสพลาทินได้

สาขาวิชาวิทยาการทางเภสัชศาสตร์ บัณฑิตวิทยาลัยมหาวิทยาลัยศิลปากร
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LIST OF ABBREVIATIONS

%	percentage
˚C	degree Celsius
<	less than
>	more than
~	approximately
®	registered trademark
µL	microliter (s)
µm	micrometer(s)
µM	micromolar (s)
µg	microgram (s)
β	beta
BSCS	*N*-benzyl-*N*,*O*-succinyl chitosan
cm^2	square centimeter
CO_2	carbon dioxide
CS	chitosan
et al.	and others
etc.	et cetera (Latin); for example, such as
g	gram (s)
GPC	gel permeation chromatography
h	hour (s)
IC	inhibition concentration
i.e.	id est (Latin); that is
L	liter (s)
M	molar (s)
MEM	modified Eagle's medium
mg	milligram (s)
min	minute (s)
mL	milliliter (s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide

mV	millivolt
MW	molecular weight
nm	nanometer (s)
NSCS	*N*-naphthyl-*N*,*O*-succinyl chitosan
OSCS	*N*-octyl-*N*-*O*-succinyl chitosan
pH	potentia hydrogenii (latin); power of hydrogen
pKa	the negative logarithm of the ionization constant of an acid
rpm	revolutions per minute or rounds per min
SM	silymarin
SM-BSCS micelles	silymarin-loaded *N*-benzyl-*N*,*O*-succinyl chitosan
SM-NSCS micelles	silymarin-loaded *N*-naphthyl-*N*,*O*-succinyl chitosan
SM-OSCS	silymarin-loaded *N*-octyl-*N*,*O*-succinyl chitosan micelles
TEM	transmittion electron microscope
UV	ultraviolet (spectroscopy)
w/w	weight by weight

CHAPTER 1
INTRODUCTION

1.1 Rational and problem statement

Cisplatin is a highly effective anticancer drug which has been used in the treatment of many solid tumors including head and neck, lung, testis, ovary and breast cancers. However, its side-effects in normal tissue, especially nephrotoxicity limit the use of cisplatin [2-4]. Cisplatin-induced nephrotoxicity is one of the most serious side effects and is presented commonly in patient. Because it is excreted by the kidney, high amount of drug may accumulate in renal cells leading to renal injury [3]. Several mechanisms of cisplatin nephrotoxicity have been reported such as the uptake of cisplatin via renal membrane transporter, p53-mediated responses, oxidative stress, inflammation, intrinsic and extrinsic apoptosis pathways, etc. [2-4]. The clinical guideline recommended for prevention of renal injury during cisplatin treatment is prehydration with normal saline solution, but renal damage still occurs [3]. In addition, numerous renoprotective approaches along the mechanism have been interested. An ideal strategy of renoprotection is to prevent kidney injury but enhance the therapeutic effects of cisplatin in cancer cells [2].

Silymarin has been known as a hepatoprotective agent and shown to be safe in animal models and human [21]. According to its therapeutic effects in liver, a lot of studies indicate that the mechanism of action may act in different pathways including antioxidant activity, cell membrane stabilizer and permeability regulator, antifibratic activity, enhanced hepatocyte regeneration and anti-inflammation [5,7,10]. Besides, hepatoprotective effect, silymarin reveals renoprotective effects via anti-inflammation, anti-oxidation to against nephrotoxic drugs such as cyclosporine, doxorubicin and cisplatin [19]. Protective effects of silymarin on cisplatin-induced nephrotoxicity were investigated in both cells line and animal models [20-21, 23]. The result indicates that silymarin shows renoprotective effects against cisplatin-induced renal damages. However, its poor aqueous solubility, poor absorption across intestinal epithelial cells,

rapid excretion restricts its efficacy. Several drug delivery approaches have been employed, such as solid dispersions, inclusion complexation with β-cyclodextrin, complexation with phosphatidylcholine, liposomes, proliposomes, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), self-emulsifying drug delivery systems (SEDDS), self-microemulsifying drug delivery systems (SMEDDS), hydrogel matrices, micelles, to improve its therapeutic effects [11,14,18]. Polymeric nano-carriers might be more interestingly because of improved permeability across the physiological barriers, increased solubility and biocompatibility [18].

Polymeric micelles present a spherical shape and are composed of inner core and outer shell [28-29,32]. Amphiphilic block copolymers containing hydrophilic and hydrophobic segments form micelles by self-assembly in aqueous medium [29,32]. The advantages over surfactant micelles are highly stable structure and low toxicity, therefore polymeric micelles have been more interesting [28-29]. Incorporation of drugs into polymeric micelles have been used for drug targeting, drug sustained release and increase of drug solubility [28]. Polymeric micelles formation depends on hydrophobic and hydrophilic interaction [39]. In general, the cohesive inner core interaction generates micelles formation by driving forces including hydrophobic force, electrostatic force, π-π interaction and hydrogen bonding. Lipophilic drugs can be into the inner core by chemical conjugation and physical entrapment. Physical entrapment can be applied to many drugs which contain hydrophobic moiety [28]. There are several physical entrapment methods that successfully prepared polymeric micelles such as dialysis, o/w emulsion, solution casting or solvent evaporation, co-solvent evaporation, solvent extraction, etc. [32-35]. In addition, forming micelles from biocompatible and biodegradable polymers might be preferred.

Chitosan is economic, biocompatible and biodegradable polymer which has been used as drug or gene carrier. Although, its applications are restricted by its insoluble property in biological solution, therefore soluble chitosan derivatives were developed [25]. Amphiphilic chitosan derivatives, *N*-benzyl-*N,O*-succinyl chitosan (BSCS), *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) and *N*-octyl-*N*-*O*-succinyl chitosan (OSCS) were synthesized by introducing hydrophobic (benzyl, octyl, naphthyl group) and hydrophilic moiety (succinyl group) into chitosan backbone. The critical micelles concentration (CMC) values of these polymers are lower than the CMC of low

molecular weight surfactants. Some studies have been reported for the successful preparation of these chitosan derivatives micelles. Poorly soluble compounds can be incorporated into hydrophobic core to increase aqueous solubility by physical entrapment methods [43-45].

Sajomsang W et al. synthesized a novel amphiphilic *N*-benzyl-*N,O*-succinyl chitosan (BSCS) through reductive N-benzylation followed by *N,O*-succinylation and developed curcumin-loaded BSCS micelles via dialysis method to increase solubility of curcumin. The BSCS consists of hydrophobic benzyl group and hydrophilic succinyl group that can form self-aggregation micelles in water during dialysis. The CMC of BSCS was 0.010 mg/mL. This result indicated that BSCS was able to form micelles in low concentration. They also characterized the physicochemical properties of BSCS micelles. Curcumin-loaded BSCS micelles showed small particle size, highly negative charges, high water solubility, strong cyclotoxicity to cervical cancer cells and high amount of drug release in physiological pH 5.5-7.4 [43].

Woraphatphadung T et al. synthesized *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) to prepare meloxicam-incorporated NSCS micelles via physical entrapment including dialysis, o/w emulsion, dropping and evaporation. The micelles properties depend on entrapment method and initial concentration of drug. NSCS micelles show less toxicity in Caco-2 cells. The release behavior of micelles was higher than pure drug [44]. Moreover, they introduced octyl groups into chitosan backbone as hydrophobic part to obtain *N*-octyl-*N,O*-succinyl chitosan (OSCS) polymer. They prepared meloxicam-loaded BSCS, NSCS and OSCS polymeric via evaporation method and evaluated the characteristics of the polymeric micelles. They found that meloxicam-loaded BSCS, NSCS and OSCS micelles exhibited different entrapment efficiency and loading capacity. The in vitro drug release was observed, the result showed that BSCS, NSCS and OSCS polymers were pH-responsive polymers which could enhance meloxicam release [45]. They concluded that NSCS micelles had a potential as meloxicam carrier for oral drug delivery [44-45].

This study aims to develop silymarin-loaded polymeric micelles based on amphiphilic chitosan derivatives (BSCS, NSCS, OSCS) to increase solubility of silymarin and to evaluate the properties of the micelles including particle size, zeta

potential, entrapment efficiency, loading capacity, drug release behavior and the effect on cancer cells and renal cells during treatment of cisplatin.

1.2 Objective of this research

1.2.1 To develop silymarin-loaded polymeric micelles based on amphiphilic chitosan derivatives for the prevention of cisplatin-induced nephrotoxicity.

1.2.2 To evaluate the influence of the physical entrapment methods, type of amphiphilic chitosan derivatives and amount of drugs on entrapment efficiency, loading capacity, particle size, zeta potential and drug release.

1.2.3 To investigate the effect of silymarin-loaded polymeric micelles on prevention of cisplatin-induced nephrotoxicity in renal cells and on cisplatin killing head and neck cancer cells.

1.3 The research hypothesis

1.3.1 The polymeric micelles based on amphiphilic chitosan derivatives can be used as silymarin carrier for prevention of cisplatin-induced nephrotoxicity.

1.3.2 The physical entrapment methods, type of amphiphilic chitosan derivatives and amount of drugs influence on the characteristics of polymeric micelles (i.e. particle size, size distribution, entrapment efficiency, loading capacity) and *in vitro* release.

1.3.3 Silymarin-loaded polymeric micelles can prevent cisplatin-induced nephrotoxicity in renal cells and cannot reduce the efficacy of cisplatin on killing head and neck cancer cells.

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2.1 Cisplatin

Cis-diamminedichloroplatinum(II), known as cisplatin, is a first platinum-containing compound which has been widely used as chemotherapeutic agent. The structure of cisplatin consists of platinum molecule that responsible for cytotoxic activity (Figure 2.1) [1]. Since cisplatin can against various types of cancers, it was approved for solid-tumors treatment including head and neck, testis, ovary, lung and breast cancer [1-4].

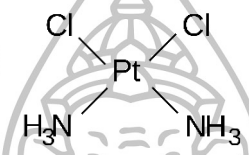

Figure 2.1 Cisplatin structure

Cisplatin represents a small molecule that can easily enter to the cells. Once cisplatin enters cell, it is activated by water molecules which replace chloride atoms. This active compound is a potent electrophile that can react with nucleophile on nucleic acid leading to DNA damage and apoptotic cell death. Moreover, one of an important mechanism of cisplatin cytotoxicity is inducing oxidative stress in cells. The formation of reactive oxygen species is result from the interaction of cisplatin with mitochondrion, leading to glutathione level decreasing, calcium homeostasis disruption and inhibition of mitochondrial function. This play an important role in cell inflammation which probably leads to apoptosis and tissue necrosis, depending on the length of exposure and concentration of cisplatin [1].

The restrict to the use of cisplatin in cancer patient treatment is the side effects in normal cells including nausea and vomiting, nephrotoxicity, cardiotoxicity ototoxicity, gastrotoxicity, myelosuppression, allergic reaction, reproductive toxicity [1-2, 4], however, the most common serious side effect of cisplatin that limit the cancer therapy is nephrotoxicity [2-4].

2.1.1 Cisplatin nephrotoxicity

Cisplatin is excreted from the body by kidney via both glomerular filtration and tubular secretion [1, 3]. The concentration of cisplatin found in kidney is about 5 times higher than serum concentration [1]. 25-35% of patients suffer from impaired renal function after treatment with cisplatin, presenting in acute renal injury, decrease in glomerular filtration, increase in serum creatinine and blood urea nitrogen, tubular damage, tubular dysfunction with salt (sodium, potassium, magnesium) wasting, proteinuria and hyperuricemia [3-5]. Since the cisplatin-induced nephrotoxicity has been reported, the use of cisplatin in cancer treatment has been limited. In addition, the risk of nephrotoxicity related to dose, frequency, cumulative dose and length of cisplatin exposure [3]. The proximal tubular cells show the high sensitivity to cisplatin because of a high mitochondrial content in the cells and the transporter that occurred in cell membrane [4]. The pathophysiological basis of cisplatin-induced nephrotoxicity has been studied for understanding of the mechanisms which may lead to the protection of renal tissue in cancer therapy [2]. Several studies revealed that cisplatin-induced nephrotoxicity involved in multiple pathways such as the uptake of cisplatin into renal cells, the potent metabolite from bioactivation of cisplatin, the interaction with mitochondrion induced excessive reactive oxygen species and interruption of cell signaling, etc. [2-5].

2.1.2 Mechanisms of nephrotoxicity

2.1.2.1. Cisplatin uptake and accumulation

Cisplatin enters to the cells via passive diffusion through the cell membrane and the uptake by cell membrane transporter named organic cation transporter 2 (OCT2) and copper transporter 1 (CTR1). Both transporters are expressed on renal proximal tubule which may play a significant role in cisplatin uptake by renal cells [2-4]. OCT2 is highly expression in renal cells leads to increasing sensitivity to cisplatin toxicity, however, OCT2 substrates such as cimetidine can decrease cisplatin uptake [2-3]. Cisplatin accumulate in renal cells due to clearance by kidney and uptake by transporters. Therefore, cisplatin-induced nephrotoxicity enlarged with dose and frequency of administration [3].

2.1.2.2. Cisplatin metabolism

After enter to renal cells, cisplatin become a more potent metabolite via bioactivation involved glutathione conjugation pathway [3]. The enzyme called γ-glutamyl transpeptidase (GGT) and aminodipeptidase were found on the proximal tubular cell surface, can transform glutathione conjugates into cysteine-conjugates which can be transported into the cells and converted to highly reactive thiols via cysteine-*S*-conjugate β-lyase [2-4].

2.1.2.3. Cellular target

Cytotoxic of cisplatin causing DNA damage is occurred via the interaction of platinum molecule and nucleophilic sites. This process inhibits cell division, resulting in cell apoptosis [1, 3]. Cisplatin also binds to mitochondrial DNA which highly content in proximal tubular cells due to the high ATP requirement of transporters, leading to mitochondrial dysfunction. Consequently, renal cells demonstrated highly sensitive to cisplatin cytotoxicity [3-4]. In addition, intercellular ATP level is decreased result from disruption of cell respiratory system [3].

2.1.2.4. Inflammation

Acute kidney injury associated with cisplatin nephrotoxicity present inflammatory responses. Proinflammatory cytokines and chemokines are induced in cisplatin treatment [2-3]. TNF-α plays an important role in inflammatory conditions. It is suggested that TNF-α is produced in kidney cells during cisplatin nephrotoxicity and related to pathological conditions [2]. Treatment with TNF-α inhibitors can decrease kidney injury from cisplatin [3]. Although, other cytokines such as IL-1β, IL-18, IL-6 and IFN-γ did not show a significant role in renal toxic associated with cisplatin [2-3].

2.1.2.5. Oxidative stress

During treatment with cisplatin demonstrated increase of various reactive oxygen species (ROS) in renal tubular cells. It is implicated that oxidative stress is one of the important factors involve in cisplatin-induced nephrotoxicity [1-2]. Since mitochondrion is a primary target of cisplatin, cisplatin-convinced

mitochondrial dysfunction results in enlargement of ROS production via respiratory chain disruption [2]. Moreover, reactive form of cisplatin can rapidly react with glutathione which is a cellular antioxidant, this process leads to accumulation of ROS in the cells [2]. Remarkably, cisplatin-induced ROS accumulation emerges to be associated with signaling pathway activation such as p38 activation and p53 activation [2]. Oxidative stress and excessive ROS in the cells can activate cell apoptosis via both extrinsic and intrinsic pathways, also induce necrosis in cells [1, 4].

2.1.2.6. Cellular mediated response

Several cell signaling or cells mediators affect to cisplatin toxicity. Cell-cycle proteins play an important role during cisplatin nephrotoxicity. The cell cycle usually regulated by the activation and inhibition of the cyclin-dependent kinase (cdk) family. A cdk inhibitor called p21 show a protective effect against cisplatin nephrotoxicity. In contrast, p53 signaling and MAPK signaling have concerned as a mediator of cisplatin-induced cell death [2-3]. p53, a well-known tumor suppressor protein, was activated by DNA damage, oxidative stress or genotoxic stress which occurred during cisplatin treatment, prior to tubular apoptosis [2-3]. The MAPKs activation consisting of extracellular signal-regulated kinase (ERK), p38, Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK) can regulate cellular homeostasis including proliferation, differentiation and apoptosis [2]. The MAPKs activation is observed in various models of cisplatin nephrotoxicity, nevertheless, the roles is still complicated.

2.1.2.7. Apoptosis and necrosis pathways

Renal tissue damage is a common histopathological characteristic which appear in cisplatin nephrotoxicity presenting in both apoptosis and necrosis form. Whereas, apoptosis cell death is preferentially observed in the use of low concentration of cisplatin, while necrosis cell death is found in a high concentration used. However, both apoptosis and necrosis are increase in kidney cells after cisplatin administration [2-3]. Apoptosis of renal tubular cells has been implicated with several pathways including the extrinsic pathway, the intrinsic pathway and the endoplasmic reticulum (ER) stress pathway [2-3]. In the extrinsic pathway, caspase-8 activation was

increased in cisplatin-induced cell death and Fas or TNF-α receptor were suggested to involved cisplatin nephrotoxicity. On the other side, the intrinsic pathway was indicated as a main apoptosis pathway during cisplatin administration because of mitochondrial disruption. It is initiated by cellular stress, apoptogenetic factors including cytochrome *c*, apoptosis-inducing factor (AIF), Smac/DIABLO, endonuclease G and others, were released from the organelles due to the porous defects on the mitochondria outer membrane. The mitochondrial injury can induce cell apoptosis. Besides, the endoplasmic reticulum (ER) stress pathway may also participate in cisplatin induced tubular cell apoptosis. Caspase-12 and calcium - independent phospholipase A_2 show an important role in this pathway [1-3].

2.1.3 Renoprotective strategies

The primary cisplatin nephrotoxicity prevention is volume expasion with normal saline solution [3, 5]. Unfortunately, even treat with a large volume hydration, renal injury associated with cisplatin toxicity still exist [3]. Therefore, various renoprotective strategies along the mechanisms have gained attention include inhibition of cisplatin uptake into the renal cells, inhibition of cisplatin metabolism, the use of antioxidant, reduction of inflammatory cytokines, protection of renal cells via cdk inhibitor, inhibition of p53 and MAPK activation, blockade of cell death pathways [2]. However, most of the studies have been investigated in cultured cells, rabbits, rats or mice, the effective in cancer patients is unclear. In addition, it is cautiously considered that inhibition of cell death pathways may reduce the efficacy of cisplatin in tumors [2]. Consequently, an imaginary renoprotective strategy is to protect renal cells but not decrease the therapeutic effects in cancer cells.

2.2 Silymarin

Silybum marianum commonly known as Milk Thistle in (Figure 2.2) Asteraceae/Compositae family, is native to Mediterranean and North Africa. It widely grows throughout Europe, America, India, China and Australia [6-7]. Milk thistle have been used as a folk medicine over thousand years for treating liver and gallbladder diseases, protection against *Amanita phalloides* mushroom poisoning [7-9]. Milk thistle is a stout looking plant with milky vein along the dark green leaves and composite vivid purple flowers. The fruits showing brown-black color contain an isomeric mixture of flavonolignan isomers named silymarin [6-7].

Figure 2.2 Milk Thistle[61,62]

Silymarin is known as hepatoprotective agent which can be extracted from seeds of milk thistle. It consists of silybin 60-70%, silychristin 20%, silydianin 10% and isosilybin 5% [7, 10-11]. The most active component among of these compounds is silybin which is synonymous with silibinin [6, 8, 10-11].

2.2.1. Structure

Silymarin is a group of flavonolignan isomers containing a common flavonolignan skeleton which consists of dihydroflavonol taxifolin linked to coniferyl alcohol moiety through an oxeran ring. Opening of oxeran ring leads to loss of biological activity [6]. The mixture of silybin and isosilybin diastereoisomers are established in the forms of silybin A, silybin B, isosilybin A and isosilybin b (Figure 2.3) [8].

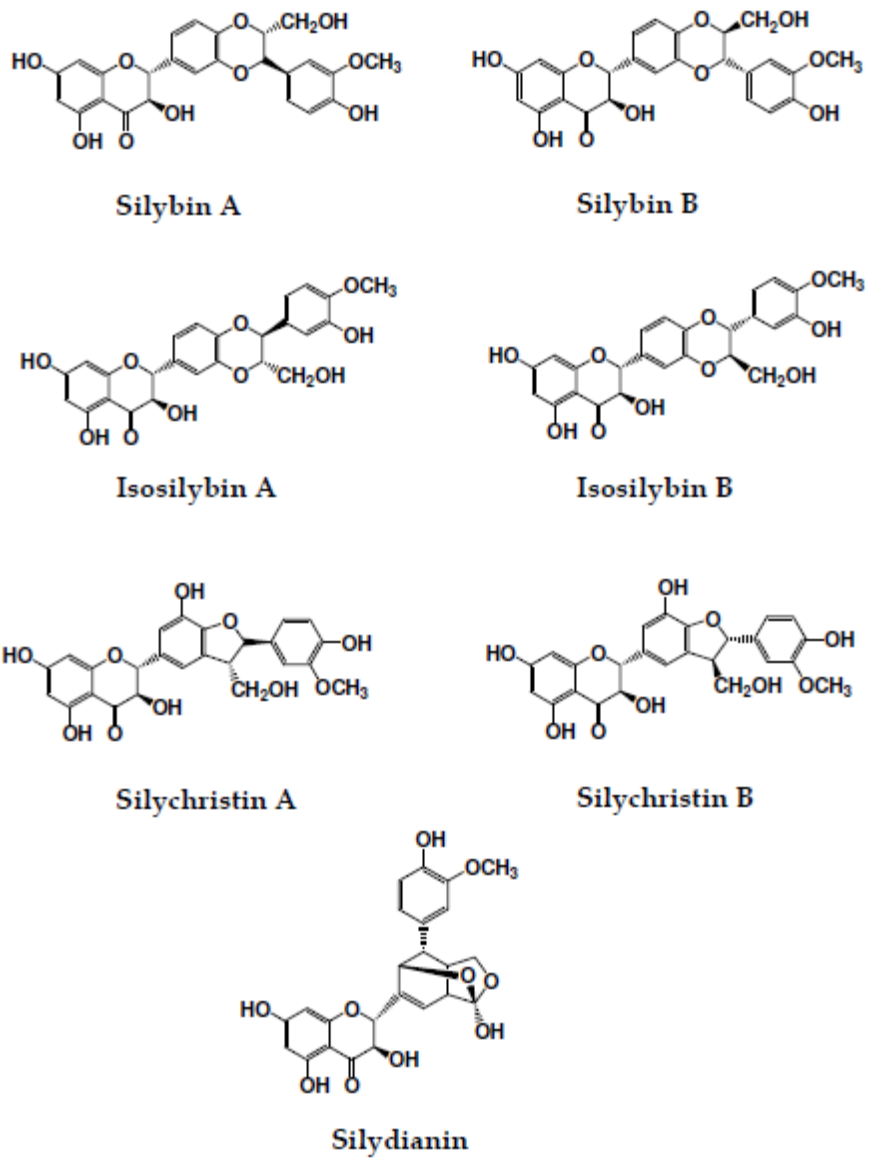

Figure 2.3 Silymarin structure[6]

2.2.2. Physicochemical and pharmacokinetic properties

Appearance of silymarin is yellow powder with characteristic odor. Degradation of silymarin is stimulated by light and oxidizing agents. Significant degradation of silymarin in water is observed at the temperature above 100°C and show first-order degradation kinetic at 140°C [12]. In the solution state, it is stable in pH 1-7 and degrades at pH 9 [13]. Low aqueous solubility of silymarin was reported to be 0.04 mg/mL [9]. Therefore, it is classified in poor solubility group.

The bioavailability of silymarin is restricted due to poor water solubility, the degradation by gastric fluid, low permeability across intestinal membrane [10, 14-15]. It is reported that only 20-50% of orally administered silymarin can be absorbed from the gastrointestinal tract [9-11, 14-16]. In addition, silymarin mostly undergoes extensive enterohepatic circulation result in excreted in bile as sulfate and glucuronide conjugates, and only less than 10% is excreted in urine [7, 9, 11, 16]. All these reasons may lead to reducing silymarin therapeutic effects.

The absorption of silymarin after oral administration demonstrated with the t_{max} of 2-4 hours and $t_{1/2}$ of 6 hours [9-16]. Both free and conjugated form of silymarin is rapidly absorbed and shows a good tissue distribution in liver, lung, stomach and pancrease [7, 9, 11].

The daily dose of silymarin is ranging from 280-800 mg/day in 2 or 3 divided dose [9, 17]. It is suggested that silymarin at dose 1,200-1,500 mg/day revealed safety and efficacy of silymarin [9]. An acute toxicity of silymarin has been studied in mice, rat, rabbit and dog [7, 17]. The oral administration tolerance is higher than intravenous infusion with the value over 10 g/kg. It is indicated that acute toxicity of silymarin is very low [17].

2.2.3. Mechanisms of action

Nowadays, silymarin has been used as a herbal medicine or dietary supplement for treating liver disorders include acute and chronic viral hepatitis, alcoholic liver diseases, cirrhosis, jaundice and against toxin-induced hepatitis [10, 14, 16]. Because of its therapeutic effects to liver, several literatures indicate the mechanism of action may act in different pathways.

2.2.2.1. Antioxidation

Silymarin acts like an antioxidant by reducing glutathione oxidation, lipid peroxidation and free radical production [7, 10, 16]. Excessive ROS in cell is caused by glutathione depletion, treatment with silymarin can increase glutathione level which can prevent DNA, RNA and others cellular component damage [7, 17]. An interaction between free radicals and unsaturated fatty acids can produce lipid peroxidation leading to degeneration of cell membrane. Moreover, silymarin appears to acts as a free radical scavenger, influences glutathione and superoxide dismutase enzymes resulting in inhibition of lipid peroxidation [7, 17].

2.2.2.2. Cell membrane stabilization

Silymarin can prevent binding of hepatotoxins to the hepatocyte cell membrane receptors leads to prevention of toxins entering to hepatocyte cells [10, 16].

2.2.2.3. Anti-inflammation

Anti-inflammatory effects of silymarin have been studied. It acts as a potent inhibitor to lipoxygenase enzyme resulting in the inhibition of leukotriene and prostaglandin production. Several studies have shown that silymarin might related to inhibition of neutrophil migration, inhibition of Kupffer cells and acts as a mast cell stabilizer [7, 17]

2.2.2.4. Antifibrotic

Formation of liver fibrosis is result from deposition of collagen fiber in liver. This process initiated by transformation of hepatic stellate cells into myofibrablast. The effect of silymarin is reduction of stellate cells proliferation which can reduce conversion of hepatic cells into myofibrablast. Finally, the result revealed that silymarin can prevent cirrhosis [7, 17].

2.2.2.5. Liver regeneration

Silymarin has ability to stimulate liver tissue regeneration via promoting ribosomal RNA polymerase leading to protein synthesis

in injured liver [7, 10, 16]. Therefore, it has been used to regenerate the liver function in alcoholic or toxin induced hepatitis.

2.2.4. Therapeutic effects

Silymarin has been widely used as a hepatoprotectant against liver disorders including viral hepatitis, drug or toxin induced hepatitis, alcoholic liver diseases, cirrhosis [10, 14, 16, 18]. Besides the hepatoprotective effects, some studies reported that silymarin has other therapeutic effects such as inhibition of carcinogenesis, antidiabetic, hypolipidemic, cardioprotective, neuroprotective, nephroprotective and anti-aging activity [18].

2.2.5. Renoprotective effects

Since silymarin has antioxidant and anti-inflammatory activity, it may also have protective effects against drug-induced nephrotoxicity. The nephroprotective effects of silymarin have been investigated in cellular studies and animals. The data revealed that silybin and silychristin can increase protein and DNA synthesis, cell proliferation rate and LDH activity in renal cells damaged from paracetamol, vincristine or cisplatin [19]. Furthermore, several studies have been shown that silymarin can against nephrotoxicity from number of drugs such as doxorubicin, aminoglycoside and cyclosporine. However, the protective effects against cisplatin-induced nephrotoxicity is the most frequently reported [19].

Abdel-Gawad S.K., et. al preformed renal protection of orally administration silymarin in male abino rats via observing the histopathological change in renal cortex. The result showed the renal cells especially tubular cells were most severely damage in cisplatin received group. The cells showed necrotic change in both proximal convoluted (PCT) tubular cells and distal convoluted (DCT) tubular cells. On the other side, the cells showed normal structure in silymarin protective group similarity to the control group, and significantly decrease in necrotic change compared with cisplatin group. This study revealed that cisplatin-induced renal damage can be prevented by pre-treatment with silymarin [20].

Abdelmeguid N.E. et. al observed the cellular and morphological change of mal Spraque Dawley rats which divided into 5 groups including control

group, vehicle solution group, cisplatin receiving group, silymarin injection post-treatment group and silymarin injection pre-treatment group. In cisplatin injected group, deceased in body weight, increased in kidney wet weight, morphology changed were observed. Otherwise, pre-treatment with silymarin before cisplatin injection present highly protective effect by decreased in histological changed. Nevertheless, the data did not show an effective protection in post-treatment with silymarin [21].

Mohamed M. et. al investigated the prophylactic effects and curative effects of silymarin on adult male rabbits which received cisplatin treatment. The rabbits were treated with oral doses of silymarin for 3 days which was given before or after a single dose of cisplatin. It was suggested that pre-treatment with silymarin as a preventive agent was more effective than post-treatment as a curative agent in term of decreased in vasculization of tubular cell cytoplasm and decreased in caspase-3 expression [22].

Tantituvanont A., et. al evaluated the mechanisms of silymarin for preventing cisplatin-induced cytotoxicity in human proximal tubular cells (HK2-cells). They investigated cell viability assay by MTT test, the scavenging activities by a flow cytometric assay, apoptosis and necrosis assay by co-staining with Hoechst 33342 and propidium iodide. Pre-treatment with silymarin could reduce cell apoptosis and necreosis induced by cisplatin, inhibit cisplatin-induced ROS generation and reduce oxidative stress in HK2-cells by scavenging activity against •OH and H_2O_2. Thus, silymarin exhibited cytoprotective effects to protect HK2-cells from cisplatin toxicity [23].

Momeni A., et. al investigated the clinical trial study to examine the nephroprotective effect of silymarin in cancer patient receiving cisplatin. The patients were given silymarin tablet 140 mg twice a day for 7 days before cisplatin administration. The serum creatinine (Cr) and blood urea nitrogen (BUN) were evaluated compared with control group who received only cisplatin. The Cr and BUN level were reduced in silymarin receiving group. This study revealed that silymarin can reduce kidney injury and nephroprotective effect cisplatin-induced nephrotoxicity in human [24].

2.2.6. Drug delivery techniques for silymarin [7,9]

Due to the major obstacles which restricted the therapeutic effects of silymarin such as poor solubility, poor permeability, rapid excretion, etc., several studies used drug delivery system to improve silymarin bioavailability. For example: (1) Increase solubility and dissolution by incorporating silymarin into hydrophilic substances by solid dispersion technique, complexation with hydroxypropyl-β-cyclodextrin [11], complexation with phosphatidylcholine, forming polymeric micelles [7,9], incorporating into Self-microemulsion drug delivery system (SMEDDs) [15-16]. (2) Designing controlled release formulation by forming hydrogel matrice, incorporating into drug carriers [18]. (3) Targeting to the active site by drug delivery system [10]. And (4) Using nanotechnology to improve the bioavailability [7,9] (Table 2.1).

Table 2.1 Drug delivery systems of silymarin.

Drug delivery approaches	Methods	Results	References
Silymarin loaded targeting liposomes	Incorporated silymarin into non-PEGylated or PEGylated liposomes with or without hepatic targeting ligand (Sito-G)	- Incorporation of silymarin into non- PEGylated liposomes with Sito-G enhanced hepatic cellular uptake - PEGylated of liposomes prevented protein adsorption and exhibited sustained release of silymarin	Elmowafy M. et al (2013) [10]

Table 2.1 Drug delivery systems of silymarin (continue).

Drug delivery approaches	Methods	Results	References
Silymarin-cyclodextrins complexation	Formed complex of silymarin with new generated β-cyclodextrins (DIMEB, RAMEB, HPBCD, QABCDP)	All new generated β-cyclodextrins could enhance silymarin solubility and bioavailability	Fenyvesi F. et al (2011) [11]
Solid dispersion and porous silica nanoparticles	Formulated a 3-day release formulation composing of silymarin solid dispersion (SD) with PVP K30, Leci, Eudragit E100 and silymarin-loaded porous silica nanoparticles (PSNs)	The formulation showed sustained release approximately 72 h via initial burst release from silymarin SD and prolong release from silymarin-loaded PSNs, improve oral bioavailability	Cao X. et al (2012) [14]
Silymarin SMEDDS	Prepared silymarin SMEDDS with Ethyl oleate, Medium chain triglyceride, Cremophor EL, Transcutol P	Silymarin SMEDDS showed high release profile and could improve silymarin solubility	Liu L., et al (2007) [15]
Silymarin SMEDDS	Formulated silymarin SMEDDS with Tween 80, Ethyl alcohol, Ethyl linoleate	Relative bioavailability of silymarin in rabbits was increased	Wu W., et al (2006) [16]

Table 2.1 Drug delivery systems of silymarin (continue).

Drug delivery approaches	Methods	Results	References
Silymarin incorporated in alginate-based hydrogel matrices	Synthesized silymarin-loaded alginate-poly (lactic-co-glycolic acid) nanoparticles (PLGA NPs) via single emulsion-solvent evaporation technique	Silymarin-loaded PLGA NPs exhibited sustained release and could improve dissolution and oral bioavailability	El-Sherbiny I.M., et al (2011) [18]
Silymarin-loaded amphiphilic chitosan polymeric micelles	Incorporated silymarin into (2-hydroxyl-3-butoxyl)-propylcarboxymethyl-chitosan (HBP-CMCHS)	Silymarin solubilized in the core of polymeric micelles and showed sustained release pattern last up to 40 h	Sui W., et al (2010) [25]
Silymarin-loaded amphiphilic chitosan polymeric micelles	Prepared Silymarin-loaded amphiphilic chitosan micelles (SM-OGC) via dialysis method	SM-OGC showed significantly increase the absorption of silymarin in the intestinal tract	Wu Y.P., et al (2009) [26]
Silymarin-loaded nanostructured lipid carriers (NLCs)	Incorporated silymarin into NLC composing of oleic acid and glyceryl monostearate via emulsification and ultrasonication	Silymarin-loaded NLCs could be transported through lymphatic pathway to avoid first pass metabolism for improving liver targeting and bioavailability	Chaudhary S. et al (2015) [40]

Wu W. et. al prepared silymarin-incorporated SMEDDs composing of tween 80, ethyl alcohol and ethyl linoleate to improve the bioavailability. The mean particle size with drug loading up to 100 mg was under 100 nm. The pharmacokinetic compared with silymarin suspension and silymarin solution were observed in rabbits. The data showed that the bioavailability of silymarin-incorporated SMEDDs was enhanced [16].

Cao X. et. al formulated a 3-day released system based on porous nanosilica. They used solid dispersion technique to increase silymarin solubility, incorporated silymarin into porous silica nanoparticle to performed controlled release system and formulated a 3-day released formulation. The pharmacokinetic in beagle dogs were observed. This study conclude that silymarin can be formulated into a 3-day released formulation to improve the bioavailability of silymarin by giving an initial burst release from silymarin solid dispersion and sustained release form silymarin-loaded porous silica nanoparticle [14].

Sui W. et. al loaded silymarin into amphiphilic chitosan derivatives polymeric micelles to increase the solubility of silymarin. They synthesized (2-hydroxyl) propyl- carboxymethylchitosan (HBP-CMCHS) as a drug carrier and prepared silymarin-loaded HBP-CMCHS polymeric micelles by physical entrapment method. The particle size, morphology and *in vitro* drug release were evaluated. The particle size of the polymeric micelles was in the range of 300 – 753 nm and the spherical shape of micelles was observed under TEM. Silymarin was incorporated into the inner core of the polymeric micelles. The drug release from the micelles showed sustained release up t0 40 h [25].

Wu Y.P. et. al investigated the absorption of silymarin-loaded amphiphilic chitosan micelles (SM-OMG) through rat intestinal compared to silymarin suspension. The absorption of SM-OMG was higher than silymarin suspension [9, 26].

Whereas, polymeric micelles are biocompatible, biodegradable and present a stable structure in nano-size, they might be more attractive to use as drug carrier [12, 27]

2.3 Polymeric micelles

Polymeric micelles are spherical-shape nanoparticles which composed of inner core and outer shell (Figure 2.4) [28-30]. Commonly, polymeric micelles contain hydrophobic segment in the inner core which means poorly soluble drug can be incorporated to improve its solubility [28-32]. Consequently, polymeric micelles have been recently interest in drug delivery systems. The core-shell structure of polymeric micelles can be formed by self-assembly of amphiphilic block copolymers or graft copolymers in aqueous media via an attractive force which produces micelle formation and a repulsive force which can prevents micelles unlimited growth [31, 33].

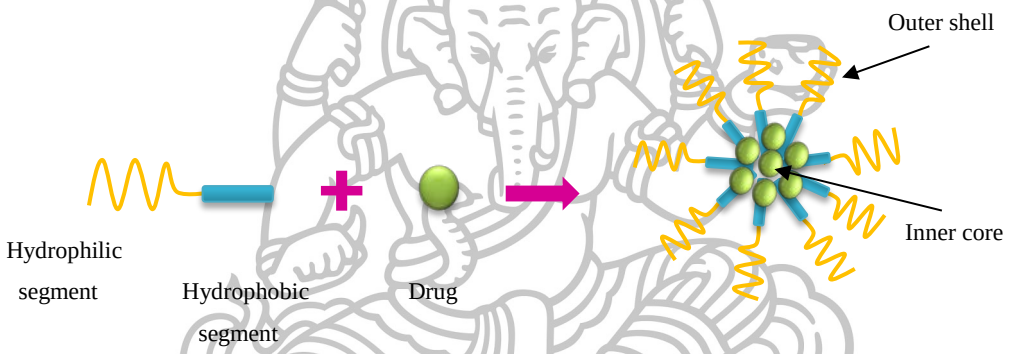

Figure 2.4 Polymeric micelles structure

2.3.1 Forming forces

The forming forces or attractive interactions present in the inner core of polymeric micelles result from the interactions of hydrophobic part in polymer chains. These interactions including hydrophobic interaction, ionic interaction, π-π interaction and H-bonding depend on the structure of polymers [28, 31, 34, 39].

2.3.1.1 Hydrophobic interaction

Hydrophobic interaction is commonly present in polymeric micelles because most of drug molecules are hydrophobic structure. Poorly soluble drug can be incorporated into the inner core of polymeric micelles through this interaction leading to increasing of drug solubility [28, 32, 34].

2.3.1.2 Ionic interaction

Electrostatic interaction or ionic interaction is generated from ionic charges of polymers and macromolecules such as DNA, RNA, Protein, amino acid, etc. Combination of macromolecules and polymeric micelles can improve drug targeting [28, 34, 39].

2.3.1.3 π-π interaction

Hydrophobic drug containing aromatic ring can produce π-π interaction which is a weak interaction but it may work cooperatively with hydrophobic interaction [28, 39].

2.3.1.4 H-bonding

H-bonding may supportively work with hydrophobic interaction [28, 39].

Polymeric micellization depends on the concentration of polymers in solvent. At the concentration below a critical micelle concentration (CMC), only single chains of polymers occur. When the concentration reached the CMC value, single polymer chains transform to micelles. Several techniques are used to forming the polymeric micelles [31, 33].

2.3.2 Preparation methods

Polymeric micelles can be prepared by various methods depending on the physicochemical properties of drugs and polymers. Drugs can be entrapped into the inner core, dispersed in polymer matrix, and adsorbed or complexed on the outer shell by physical entrapment and chemical conjugation [28, 31, 34].

2.3.2.1 Physical entrapment methods

Types and solubility of both polymers and drugs are considered to select the polymeric micelles forming techniques. Organic solvents are common used in polymeric micelles preparation because the hydrophobic segment which usually exist in polymer chain or drug molecule are not readily soluble in aqueous medium [31-34].

2.3.2.1.1 Dialysis

The dialysis method composed of solubilizing both drug and polymer in a water-miscible solvent, replacing the solvent with aqueous medium. The mixture can be dialyzed against water and the micelles formation is induced by solvent removal process. This technique applies to amphiphilic polymers and drugs which both are soluble in the water-miscible solvent [31 -34]. The common organic solvent frequently use are dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), acetronitrile, acetone, dimethylacetamide and tetrahydrofuran (THF) [32].

2.3.2.1.2 O/W emulsion method

The O/W emulsion method is prepared by dissolving the drug in a water-immiscible volatile solvent and polymer in aqueous solution. The drug in organic solvent is added to the polymer solution in ordered to form an emulsion. The drug-loaded polymeric micelles can be formed as the solvent evaporates. The use of volatile organic solvents (dichloromethane, chloroform, ethyl acetate) which are potentially toxic has been concerned [31-33, 35].

2.3.2.1.3 Evaporation and sonication/Solvent-casting method/ Mechanical dispersion method

This technique consists of dissolving polymer and drug in organic volatile solvent or a blend of solvents followed by evaporating of the solvent, the thin film of polymer and drug are remained. Rehydrating the film with a heated aqueous medium under agitation or sonication induced the formation of polymeric micelles [32-34, 36].

2.3.2.1.4 Cosolvent evaporation/Solvent displacement method

In this method, the drug and polymer are dissolved in a volatile water-miscible organic solvent (methanol, ethanol, acetronitrile, acetone, THF). The solution is injected into an aqueous medium with surfactant or stabilizer under constant stirring, the organic solution may rapidly diffuse through the aqueous medium to form the micelles. Moreover, water can be added into the organic solution to forced micellization of polymer. The organic solvent is removed by evaporation [33, 36].

2.3.2.2 Chemical conjugation method

For chemical conjugation, the drug molecule form chemical bond with functional group of polymer chain. The drug release from polymeric micelles by cleavage of bond. However, both released form and conjugated form are existed [28].

2.3.3 Characterization

2.3.3.1. Determination of critical micelle concentration (CMC)

Critical micelles concentration (CMC) value is an important factor presenting the micelle forming. The unimers begin to turn into polymeric micelles while the concentration of polymers raise above CMC value [37-38]. Polymeric micelles show more stability than low molecular weight surfactant micelles because of the significantly lower CMC value [29, 37]. The CMC can be measured by several methods, pyrene fluorescent probe is the most widely used [31]. Pyrene is a hydrophobic molecule which sensitive to surrounding polarity [31]. At the concentration below the CMC, pyrene is solubilized in high polarity medium leading to low fluorescence intensity of pyrene. When the core forming of polymeric micelles is occurred, pyrene participate into the hydrophobic core resulting in increasing fluorescence intensity [29, 31]. The extremely change of the intensity are observed when the concentration are reaching CMC value. The fluorescence intensity ratio from emission spectra of pyrene are plot to determine the CMC value [31].

2.3.3.2. Micellar size

The micellar size is affected by several factors such as molecular weight of polymer, relative proportion of hydrophilic and hydrophobic chains, aggregation number, organic solvent used, preparation methods, etc. [31]. The micellar diameter and size polydispersity index can be measured by dynamic light scattering (DLS). Moreover, the size and micellar shape can be observed via microscopy methods including atomic force microscopy (AFM), transmittion electron microscope (TEM) and scanning electron microscope (SEM) [31].

2.3.3.3. Zeta potential

Zeta potential is measured to confirm that polymeric micelles have negative or positive charges. Polymeric micelles with highly charges are more stable due to it can prevent micellar aggregation [29].

2.3.3.4. Structure stability

The physical stability of polymeric micelles is usually evaluated by gel permeation chromatography (GPC) due to single chains and micelle particles exhibit different elution time. The structural stability of polymeric micelles can be determined by their elution in aqueous media through the size exclusion column [31].

2.3.3.5. Drug release

Drug release from polymeric micelles depends on the strength of cohesive forces between the drug and the inner core segments, pH-sensitive bonds, dilution, ionic strength in the medium, steric hindered of the shell, etc. [32].

2.3.4 Advantages

2.3.4.1. Highly stable structure

The high structural stability of polymeric micelles result from the entanglement of polymer chains. Two perspective were used to described the stability of polymeric micelles. The static stability arises from the low CMC value of polymeric micelles which simply form self-aggregation, the dynamic stability relates to the low dissociation rates of the polymeric micelles [28].

2.3.4.2. Separated functionality

Two separated phases of polymeric micelles act as different roles in the system. The inner core of the structure is used for entrapping drug molecules but on the other hand the outer shell is responsible for determination of drug distribution and pharmacokinetic behavior [28]. Additionally, modification of the outer shell with targeting ligand can enhance drug uptake in the targeted cell leading to improve efficacy.

2.3.4.3. Low toxicity

Polymeric micelles are less toxicity than low molecular weight micelles [28]. Since polymeric micelles are formed by hydrophobic forces, the micelles can be broke and release as single polymer chains leading to the complete excretion from the body [28].

2.3.5 Applications

2.3.5.1. Drug solubilization

Since most of drugs are water-insoluble molecules, surfactants or drug delivery systems are used to increase drug solubility in aqueous media [28]. Poorly water-soluble compound can be incorporated into the hydrophobic inner core of polymeric micelles through cohesive interactions. The micelles capacity depends on structural compatibility of drug molecule and hydrophobic segment of polymer [32, 38]. Furthermore, incorporation of drug into polymeric micelles exhibits lower toxic compared with low molecular weight surfactants and diminish toxic side effects of anticancer drugs [28]. This indicated that polymeric micelle is a good choice for using as drug delivery system.

2.3.5.2. Passive drug targeting

Due to the structure of polymeric micelles contain hydrophilic shell which can prevent the macrophages uptake and their high molecular weight can prevent renal excretion, resulting in prolong blood circulation [28, 31]. They show lower bio-distribution in normal tissue but in contrast higher accumulation in target cells especially tumor cells, therefore they can increase efficacy and decrease toxicity [31].

2.3.5.3. Active drug targeting

Introducing targeting ligand to the outer shell of polymeric micelles or synthesizing pH/thermo-responsive polymer can enhance drug delivery to the target site [31].

2.3.5.4. Sustained release

Control of drug release from polymeric micelles is associated with several parameters including hydrophobicity, viscosity of the hydrophobic inner core, diffusion and partition coefficient of the drug [28]. The release of drug occurs via polymeric micelles dissociation to free polymer chains [27]. The polymer can be designed to optimize drug release site and rate for delivery to the target [28].

2.4 Chitosan

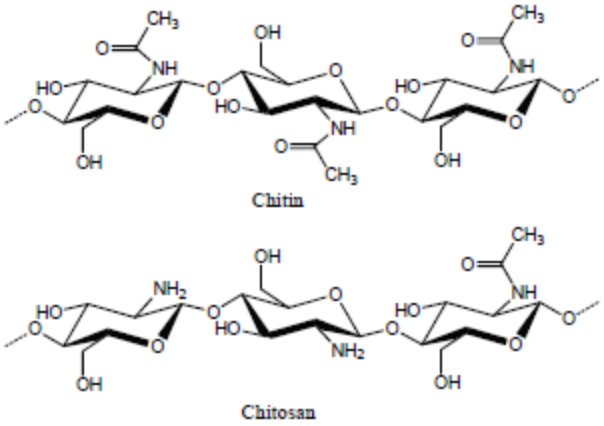

Figure 2.5 Chitin and Chitosan structure[41]

Chitosan is a biopolymer result of deacetylation of chitin which found in crustacean shells [41-42]. The structure of chitosan consists of D-glucosamine and N-acetyl-D-glucosamine which is obtained in linear chain [41-42]. Chitosan show great advantages in pharmaceutical and biomedical applications because of its biocompatibility, biodegradability, non-toxicity. Moreover, several drug delivery systems such as hydrogels, films, fibers, nanoparticles are developed from chitosan or chitosan derivatives [41].

Due to chitosan contains amino groups (pKa 6.2-7.0) in the structure resulting in insoluble in water or aqueous bases [41]. Chemical modification of chitosan structure including graft reactions, ionic interactions with various functional groups are used for improving chitosan solubility or utilizing in biomedical and pharmaceutical fields [42]. The chemical reaction used for synthesizing chitosan derivatives may relate to reductive amination of -NH_2 groups at C-2 position and

esterification or etherification of -OH group at C-3 or C-6 position [42]. Some chitosan derivatives were successfully synthesized such as O-and N-Carboxymethlchitosans, Chitosan 6-O-sulfate, N-methylene phosphonic chitosans, Trimethylchitosan ammonium, Carbohydrate branched chitosans, Chitosan-grafted copolymers, alkylated chitosans, etc. [42].

2.4.1. Pharmaceutical and biomedical applications [41, 42]

2.4.1.1. Tissue engineering

2.4.1.2. Would healing/would dressing

2.4.1.3. Burn treatment

2.4.1.4. Artificial skin

2.4.1.5. Surgical sutures

2.4.1.6. Dental implants

2.4.1.7. Bone rebuilding

2.4.1.8. Drug delivery systems

2.5 Novel pH-responsive polymeric micelles based on amphiphilic chitosan derivatives

Sajomsang W. et al. synthesized a novel amphiphilic chitosan derivative namely *N*-benzyl-*N*,*O*-succinyl chitosan (BSCS) via reductive *N*-benzylation and *N*,*O*-succinylation to prepared pH-responsive polymeric micelles for oral curcumin delivery. They characterized BSCS polymer by H^1-NMR and FTIR to confirmed that it is successfully introduced both benzyl groups and succinyl group into chitosan backbone. Since BSCS contain hydrophilic moieties (succinyl groups) and hydrophobic moieties (benzyl group), it can self-aggregate to form micelles in water. Blank and curcurcumin-loaded polymeric micelles were prepared by dialysis method. The micellar size was less than 100 nm and presented in spherical shape. The *in vitro* release behavior of curcumin from BSCS was performed under various pH medium compared to free curcumin. The result showed that the release rate of curcumin-loaded BSCS micelles was significantly higher than free drug at pH 5.5, 6.8, 7.4. On the other hands, the release of curcumin in pH 1.2 (both free drug and curcumin-loaded BSCS micelles) was slow and less than 25% within 10 h. It is revealed that BSCS micelles

were pH-responsive polymeric micelles which were potentially drug delivery of hydrophobic drug [43].

Woraphatphadung T et al. prepared meloxicam-loaded *N*-naphthyl-*N*,*O*-succinyl chitosan (NSCS) via various physical entrapment methods include dialysis, o/w emulsion, dropping and evaporation. The polymeric micelles prepared by different methods and various amount of initial drug presented dissimilar properties in term of entrapment efficiency, particle size and structural stability characterized by GPC. The release profile of meloxicam from polymeric micelles was performed in pH changed medium. At the pH 1.2 in first 2 h, meloxicam releasing from free drug and polymeric micelles were not difference, less than 20%. When the pH was changed to 6.8, higher amount of meloxicam was release from polymeric micelles compared to free drug. It is indicated NSCS micelles have a potential as meloxicam carrier for oral drug delivery [44].

Woraphatphadung T et al. synthesized various amphiphilic chitosan derivatives via reductive *N*-amination and *N*,*O*-succinylation to obtained *N*-benzyl-*N*,*O*-succinyl chitosan (BSCS), *N*-naphthyl-*N*,*O*-succinyl chitosan (NSCS) and *N*-octyl-*N*-*O*-succinyl chitosan (OSCS) (Figure 2.6). The H^1-NMR and FTIR spectra indicated that it is successfully introduced benzyl groups, naphthyl groups, octyl groups and succinyl groups into chitosan backbone.

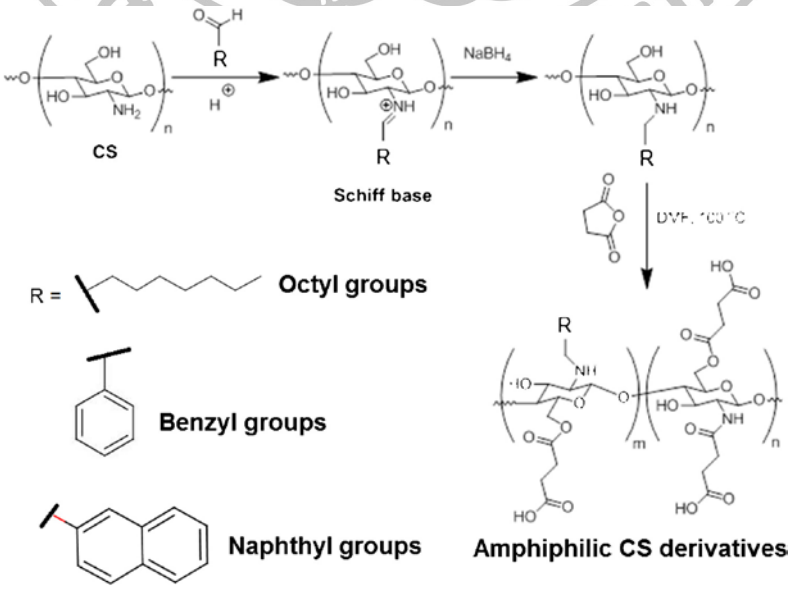

Figure 2.6 Amphiphilic chitosan derivatives synthesis[45]

These polymers can form polymeric micelles by self-aggregation in aqueous media. The CMC value was determined by fluorescence spectroscopy with pyrene probe, found to be 0.0855, 0.0678 and 0.0575 mg/mL for BSCS, NSCS and OSCS, respectively. The results showed the lower CMC value compared to low molecular weight surfactants. They used meloxicam as a hydrophobic model drug to incorporate in polymeric micelles. Meloxicam-loaded BSCS, NSCS and OSCS micelles were prepared by solvent evaporation method with various amount of initial drug (0-40%wt). The entrapment efficiency and loading capacity was affected by different types of polymers while OSCS micelles showed highest entrapment efficiency and loading capacity. The atomic force microscopy (AFM) images exhibited different shape and size of the micelles in various pH medium. At pH above pKa, the micelles will be dissociating and swelling leading to the release of drug from the micelles. Since all polymers formed pH-responsive micelles, the release behavior was not different but higher than free drug. The permeation study was performed in porcine intestinal membrane, the permeation rates of meloxicam-loaded polymeric micelles and free drug were not different because of its high permeability. Additionally, the cytotoxicity of BSCS, NSCS and OSCS micelles in Caco-2 cells were observed by MTT test. The cell viability percentage showed that all polymers were low toxicity which can be used as potential oral drug delivery system [45].

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Materials for preparing polymeric micelles

3.1.2. Reagents for HPLC analysis

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3.1.4. All other chemicals

3.2. Equipments

3.3. Methods

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3.3.1.1. Preparation of silymarin-loaded polymeric micelles via various physical entrapment methods

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3.3.2. Entrapment efficiency (%EE) and loading capacity

3.3.3. Quantitation of silymarin by HPLC analysis

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3.3.7. Cell culture and maintenance

3.3.7.1. Preparation of culture media

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3.3.7.3. Subculturing

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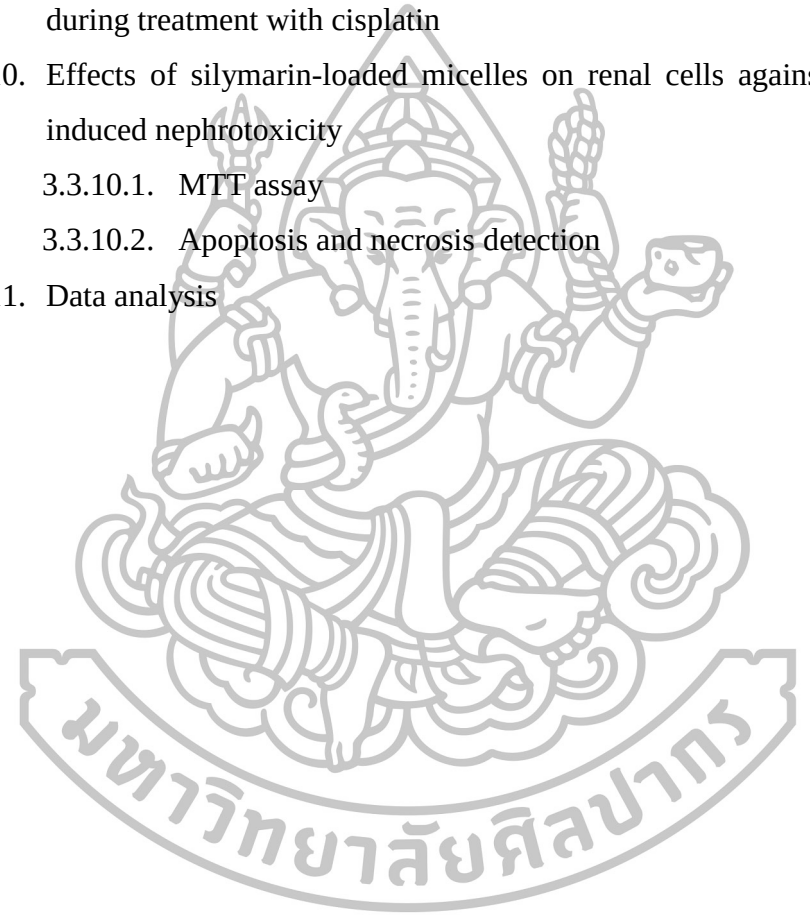
3.3.9. The effects of silymarin-loaded micelles on head and neck cancer cells during treatment with cisplatin

3.3.10. Effects of silymarin-loaded micelles on renal cells against cisplatin-induced nephrotoxicity

3.3.10.1. MTT assay

3.3.10.2. Apoptosis and necrosis detection

3.3.11. Data analysis



3.1 Materials

3.1.1 Materials for preparing polymeric micelles

a. Silymarin (Sigma-Alrich)

b. N-benzyl-N,O-succinyl chitosan (BSCS) (NANOTECH)

c. N-naphthyl-N,O-succinyl chitosan (NSCS) (NANOTECH)

d. N-octyl-N-O-succinyl chitosan (OSCS) (NANOTECH)

e. Dimethyl sulphoxide (DMSO) (Fisher Scienntific)

f. Dimethylformamide (DMF) (QRëC)

g. Acetone (QRëC)

3.1.2 Reagents for HPLC analysis

3.1.2.1 Methanol HPLC grade (Honeywell, QRëC)

3.1.2.2 Phosphoric acid 85% (Merk)

3.1.3 Tissue culture reagents

3.1.3.1 DMEM (GIBCO™)

3.1.3.2 DMEM/F12 (GIBCO™)

3.1.3.3 Fetal bovine serum (GIBCO™)

3.1.3.4 Penicillin/Streptomycin

3.1.3.5 Apotransterin

3.1.3.6 Insulin

3.1.3.7 Selenite sodium

3.1.3.8 EGF

3.1.3.9 Hydrocortisone

3.1.4 All other chemicals

3.1.4.1 Hydrochloric acid (QRëC)

3.1.4.2 Trisodium phosphate (CARLO ERBA)

3.1.4.3 Sodium chloride (Sigma-Alrich)

3.1.4.4 Potassium chloride (Univar)

3.1.4.5 Sodium hydroxide (Univar)

3.1.4.6 Disodium hydrogen phosphate (Univar)

3.1.4.7 Potassium dihydrogen phosphate (QRëC)

3.1.4.8 Sodium lauryl sulfate (Fisher Scienntific)

3.1.4.9 Hoechst 33342

3.1.4.10 Propidium iodide (Sigma-Alrich)

3.1.4.11 0.25% Trypsin-EDTA (GIBCO™)

3.1.4.12 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT (Sigma-Aldrich□, MO, USA)

3.2 Equipments

3.2.1 Beaker (Pyrex, USA)

3.2.2 Glass bottle

3.2.3 Analytical balance (Satorius CP224S, Scientific promotion Co., Ltd.)

3.2.4 Syringe 1 mL, 10 mL, 50 mL

3.2.5 1.5 ml, 2 ml Eppendrof® tubes

3.2.6 Micropipette 2-20 µL, 10-100 µL, 20-100 µL, 100-1000 µL, 1-5 mL (Masterpette®; Bio-Active Co., Ltd.) and micropipette tip

3.2.7 Dialysis bag (CelluSep® 6000-8000 MWCO Membrane Filtration Products, USA).

3.2.8 10 ml test tube

3.2.9 Zetasizer Nano ZS (Malvern instruments Ltd., Malvern, UK)

3.2.10 Magnetic stirrer and magnetic bar

3.2.11 N_2 gas

3.2.12 Larminar hood

3.2.13 Bath sonicator

3.2.14 Probe sonicator (Sonics VibraCellTM)

3.2.15 15 mL, 50 mL centrifuge tubes-sterile (Biologic research company)

3.2.16 Centrifuge (ALC Multispeed centrifuge, PK 121R)

3.2.17 Refrigerator 4 °C, -20 °C and -80 °C

3.2.18 Vortex mixer

3.2.19 Thermometer

3.2.20 Water bath (Hetofrig CB60; Heto High Technology)

3.2.21 Duran bottle 500 mL, 1 L, 2 L

3.2.22 High performance liquid chromatography (HPLC)

3.2.23 HPLC vial

3.2.24 5µ C18 HPLC column (phenomenex®, USA)

3.2.25 Cellulose acetate filter 0.45 µm

3.2.26 Sartorius® filter set (Sartorius BORO 3.3 Goettingen, Germany)

3.2.27 Peristatic pump on the suction line

3.2.28 pH meter (HORIBA compact pH meter B-212)

3.2.29 pH meter (Mettler Toledo; Switzerland)

3.2.30 Shaking incubator (GFL 3031)

3.2.31 Franz diffusion cell

3.2.32 Automatic autoclave (Model: LS-2D; Scientific promotion CO., Ltd.)

3.2.33 25 cm^2 and 75 cm^2 cell culture flask (Corning®; Corning Incorporated)

3.2.34 96-well cell culture cluster (Costar®; Corning Invorporated)

3.2.35 CO_2 incubator (HERA Cell 240 Heraeus)

3.2.36 Laminar air flow (BIO-II-A)

3.2.37 Pipette aid (Powerpette Plus; Bio-Active Co., Ltd.)

3.2.38 Aluminium foil

3.2.39 Parafilm

3.2.40 Microplate reader

3.2.41 Microscope

3.2.42 Desiccator

3.3 Methods

3.3.1 Preparation of polymeric micelles with and without silymarin

3.3.1.1. Preparation of silymarin-loaded polymeric micelles via various physical entrapment methods

3.3.1.1.1 Dialysis method

Silymarin (20%w/w to polymer) was dissolved in dimethyl sulphoxide (DMSO), the 1 mL solution was added to 5 mg of *N*-benzyl-*N*,*O*-succinyl chitosan (BSCS). The mixture was diluted with DMSO to a final volume of 2 mL and stirred until clear solution was formed. The clear solution was then placed in a dialysis bag and dialyzed against distilled water for 24 h. Then the mixture was centrifuged at 1000 rpm for 5 min and the supernatant was collected.

3.3.1.1.2 Evaporation and sonication method

Silymarin (20%w/w to polymer) was dissolved in dimethylformamide (DMF), the 0.1 mL solution was added to 5 mg of BSCS and diluted with 0.3 mL DMF to a final volume of 0.45 mL. A 0.15 mL of acetone was added to the mixture and stirred under nitrogen gas flow until the solvent was completely evaporated. A 3 mL of distilled water was added then a probe-type sonicator was used to sonicate the mixture at 80°C for 20 min (sonication time of 5 min and a stand by time of 5 min for 2 cycles). Then the mixture was centrifuged at 1000 rpm for 5 min and the supernatant was collected.

3.3.1.1.3 Dropping method

Silymarin (20% w/w to polymer) was dissolved in 0.5 mL DMSO, the solution was added to 5 mg of BSCS. The mixture was slowly dropped into 2.5 mL of stirred water and stirred at the room temperature for 24 h. The mixture was placed in a dialysis bag and dialyzed against distilled water for 24 h. Then the mixture was centrifuged at 1000 rpm for 5 min and the supernatant was collected.

3.3.1.1.4 Cosolvent evaporation method

Blank micelles were prepared by dissolving BSCS 5 mg in DMSO 2 mL and stirred until clear solution was formed. The clear solution was then placed in a dialysis bag and dialyzed against distilled water for 24 h. The blank micelles was collected and diluted with water to a final volume of 3 mL. Silymarin (20% w/w to polymer) was dissolved in acetone, the solution was injected into 3 mL of the stirred blank micellar solution. The mixture was stirred at the room temperature for 24 h then stirred at 70°C for 15 min until acetone was completely evaporated. Then the mixture was centrifuged at 1000 rpm for 5 min and the supernatant was collected.

3.3.1.2 Preparation of polymeric micelles with different weight ratios of silymarin

The preparation method resulting high silymarin entrapment efficiency and loading capacity (evaporation and sonication method, cosolvent evaporation) was selected to prepare the polymeric micelles with 3 types of polymers (BSCS, NSCS, OSCS) and different weight ratios of silymarin (0 - 60%w/w to polymers).

3.3.2 Entrapment efficiency (%EE) and loading capacity

The amount of silymarin in polymeric micelles prepared by each method was measured using high performance liquid chromatography (HPLC). Silymarin-loaded polymeric micelles was dissolved the in a mixture of DMSO:H_2O at a 9:1 volume ratio then filtered through a syringe filter membrane (0.45 μm pore size) [44-45]. The average amount of silymarin was determined in triplicate. The % entrapment efficiency (%EE) and loading capacity (LC) were calculated by the following equations:

$$\%EE = \frac{\text{The amount of determined silymarin in micelles}}{\text{Initial amount of silymarin used for the preparation}} \times 100 \quad (1)$$

$$LC = \frac{\text{The amount of determined silymarin in micelles}}{\text{Amount of graft copolymer used for the preparation}} \quad (2)$$

3.3.3 Quantitation of silymarin by HPLC analysis

The amount of silymarin was determined using high performance liquid chromatography (HPLC, Agilent 1100 series) using a 5μm C18 HPLC column (phenomenex®, USA) with gradient mobile phases. The quantitative determination of the amount of silymarin in the sample was obtained from the calibration curve.

Gradient mobile phases [60]

Solvent A : water:methanol:85% phosphoric acid (80:20:5)

Solvent B : water:methanol:85% phosphoric acid (20:80:5)

Condition [60]

Flow rate : 1.0 mL/min

Injected volume : 10 μL

UV detection : 288 nm

3.3.4 Characterization of micelles

3.3.4.1 Particle size and zeta potential

The sample was diluted with water at a 1:100 volume ratio. The average particle size, size distribution (polydispersity index; PDI) and zeta potential of the blank micelles and silymarin-loaded polymeric micelles were determined in triplicate at 25 °C using a Nano Zetasizer (Malvern, Worcestershire, UK).

3.3.4.2 Morphology

The morphology of blank polymeric micelles and silymarin-loaded polymeric micelles was observed by transmission electron microscopy (TEM).

3.3.4.3 Gel permeation chromatography (GPC)

The structure stability of polymeric micelles was analyzed using a HPLC system (Agilent 1200 series, USA) equipped with a Shodex® GFC SB804HQ column. 50 μL of polymeric micelles was filtrated through a 0.45 μm filter membrane then

injected into the column, operating with distilled water at the flow rate of 1.0 mL/min and detection by refractive index (RI) and UV/Visible detector.

3.3.5 *In vitro* drug release study

The release of silymarin from polymeric micelles in 0.1 N HCl with NaCl, pH 1.2 changed to pH 6.8 with trisodium phosphate condition and phosphate buffer (PBS) pH 7.4 solution was investigated using dialysis bag method. Silymarin-loaded polymeric micelles or silymarin suspension were placed in the dialysis bag and immersed in the medium under constant shaking at 100 rpm at 37°C. At time intervals, 1 mL of each sample was collected, and 1 mL of fresh medium was added. The amount of silymarin in the sample was analyzed by HPLC. All experiments were performed in triplicate.

3.3.6 *In vitro* permeation study

The *in vitro* permeation across porcine intestine study was performed using Franz diffusion cells. Silymarin suspension or silymarin-loaded polymeric micelles was adjusted pH to 6.8 and then was added into the donor compartment and PBS (pH 7.4) with 2% sodium lauryl sulfate solution was added into the receiver compartment. The operation was controlled by circulating-water jacket at 37°C under constant stirring. At time intervals, 1 mL of each sample was withdrawn from the receptor chamber and fresh medium was added to replace. The amount of silymarin was determined in triplicate using HPLC.

3.3.7 Cell culture and maintenance

3.3.7.1 Preparation of culture media

3.3.7.1.1. Head and neck cancer cells (HN22)

DMEM medium powder and 2.2 g of $NaHCO_3$ was dissolved in 900 mL of sterile water and sodium hydroxide solution or hydrochloric solution was added to adjust the pH to desired pH (pH 7.4). Sterile water was added to adjust to the final volume of 1,000 mL then filtered through 0.22 μm membrane filter set. The medium was supplement with fetal bovine serum (FBS) and penicillin/streptomycin solution.

3.3.7.1.2. Renal cells (RPTEC/TERT1)

DMEM/F12 medium powder and 3.7 g of $NaHCO_3$ was dissolved in 900 mL of sterile water and sodium hydroxide solution or hydrochloric solution was added to adjust the pH to desired pH (pH 7.4). Sterile water was added to adjust to the final volume of 1,000 mL then filtered through 0.22 μm membrane filter set. The medium was supplemented with fetal bovine serum (FBS), penicillin/streptomycin solution, apotransterin, insulin, selenite sodium, EGF and hydrocortisone.

3.3.7.2 Cultivation

Head and neck cancer cells and renal cells were cultured in completed growth media and incubated at 37 °C and 5% CO_2. Cultivated cells were observed using an inverted microscope.

3.3.7.3 Subculturing

Cultivated cells were observed under an inverted microscope. In case of confluency of cultivated cells obtained 80-90% in tissue culture flask, the medium was withdrawn and the cells were rinsed with PBS. 0.25% trypsin/EDTA solution was added and incubated at 37 °C and 5% CO_2 for approximately 10-15 minutes for RPTEC/TERT1 cells and 3-5 minutes for HN22 cells until the cells floated. Excess serum-containing medium was added to obtained cells suspension then transferred to a sterile centrifuge tube and centrifuged at 1,000 rpm for 3 minutes. The medium was withdrawn and replaced with fresh completed medium. 1 mL of the cells suspension was added to a new tissue flask and complete medium was added for split ratio 1:3 for renal cells and 1:5 to 1:10 for head and neck cancer cells. The subcultured cells were incubated at 5% CO_2, 37°C for cultivation.

3.3.8 Cytotoxicity evaluation

The cell viability of HN22 cells and RPTEC/TERT1 cells was investigated using the MTT assay. The cells were cultured in a 96-well plate in 100 µl of medium, incubated at 37 °C and 5% CO_2. Then, the media was replaced with various concentrations of cisplatin, free silymarin, blank polymeric micelles and silymarin-loaded polymeric micelles and incubated for 24 h (HN22) or 72 h (RPTEC/TERT1). After incubation time, the cells were rinsed with PBS and incubated in MTT-containing medium (1 mg/ml) for 4 h. 100 µl of DMSO was added to dissolve formazan crystals. The absorbance was measured at 530-550 nm using a microplate reader. Relative cell viability (%) was calculated compare with non-treated cells.

3.3.9 Effects of silymarin-loaded micelles on head and neck cancer cells during treatment with cisplatin

HN22 were cultured in a 96-well plate for 24 h. The pre-treatment or co-treatment of various concentrations of silymarin or silymarin-loaded micelles were performed. After 1 h, cisplatin solution was added into the well-plate of pre-tratment cells and incubated for 24 h. After incubation time, the cell viability was measured by MTT assay calculated compared with non-treated cells.

3.3.10 Effects of silymarin-loaded micelles on renal cells against cisplatin-induced nephrotoxicity

3.3.10.1 MTT assay

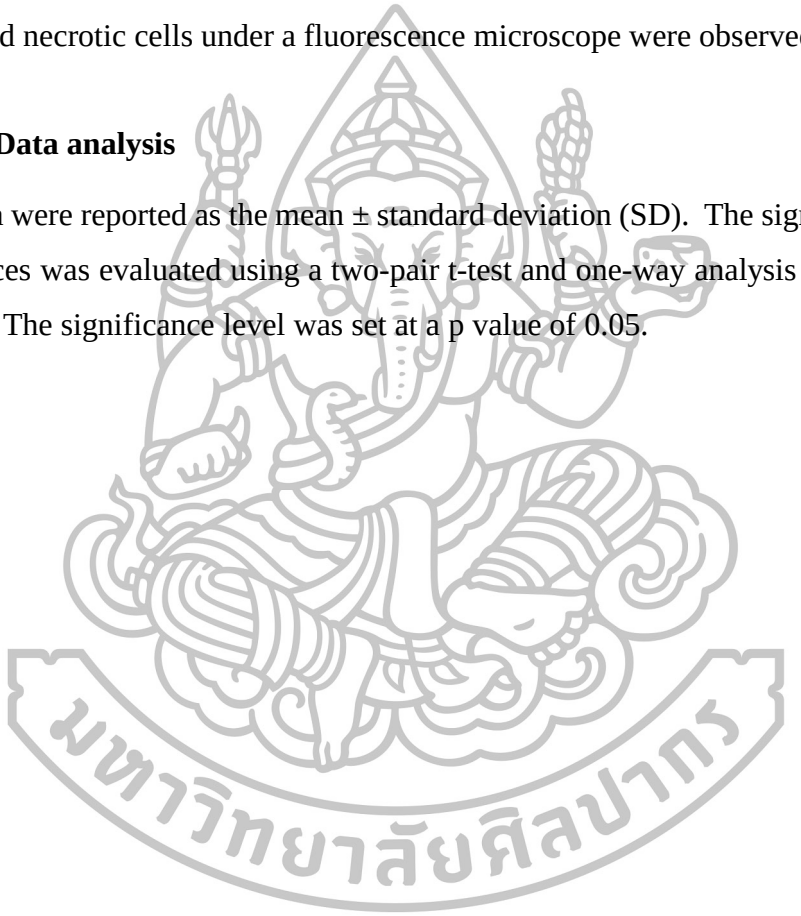
RPTEC/TERT1 cells were cultured in a 96-well plate in 100 µl of medium, incubated at 37 °C and 5% CO_2. Then, the media were replaced with the pre-treatment of various concentrations of silymarin or silymarin-loaded micelles. After 1 h, cisplatin solution was added into well-plate and incubated for 72 h. After incubation time, the cell viability was measured by MTT assay calculated compared with non-treated cells.

3.3.10.2 Apoptosis and necrosis detection

RPTEC/TERT1 cells were cultured in a 48-well plate, then pre-treated with various concentrations of silymarin or silymarin-loaded polymeric micelles for 1 h and cisplatin solution was finally added, incubated at 37 °C and 5% CO_2. After 72 h, the cells were washed with PBS and incubated with Hoechst 33342 10 μg/mL and propidium iodide 5 μg/mL in PBS for 20 min. The cells were washed with PBS, then apoptotic and necrotic cells under a fluorescence microscope were observed

3.3.11 Data analysis

All data were reported as the mean ± standard deviation (SD). The significance of the differences was evaluated using a two-pair t-test and one-way analysis of variance (ANOVA). The significance level was set at a p value of 0.05.



CHAPTER 4
RESULTS AND DISCUSSION

4.1 Polymeric micelles preparation

4.1.1 Effects of entrapment methods

4.1.2 Effects of different weight ratios of silymarin and various types of polymers on polymeric micelles properties

4.1.2.1 Entrapment efficiency and loading capacity

4.1.2.2 Particle size and zeta potential

4.1.2.3 Morphology and structure stability

4.2 *In vitro* drug release

4.2.1 pH 1.2 changed to 6.8

4.2.2 pH 7.4

4.3 Permeation study

4.4 Cytotoxicity

4.4.1. Head and neck cancer cells (HN22)

4.4.1.1. Free SM

4.4.1.2. Blank polymeric micelles

4.4.1.3. SM-loaded polymeric micelles

4.4.2. Renal cells (RPTEC/TERT1)

4.4.2.1. Free SM

4.4.2.2. Blank polymeric micelles

4.4.2.3. SM-loaded polymeric micelles

4.5 Effects of silymarin-loaded micelles on head and neck cancer cells during treatment with cisplatin

4.5.1. Cisplatin cytotoxicity

4.5.2. Effects of free SM and SM-loaded polymeric micelles pre-treatment

4.5.3. Effects of free SM and SM-loaded polymeric micelles co-treatment

4.6 Effects of silymarin-loaded micelles on renal cells against cisplatin-induced nephrotoxicity

4.6.1. MTT assay

4.6.1.1 Free SM

4.6.1.2 SM-loaded BSCS micelles

4.6.2. Apoptosis and necrosis detection

4.1 Polymeric micelles with or without silymarin preparations

4.1.1 Effect of entrapment methods

Amphiphilic chitosan can form polymeric micelles by self-aggregation via attractive forces among hydrophobic segments [31,33]. Amphiphilic chitosan *N*-benzyl-*N,O*-succinyl chitosan (BSCS) was used to investigate the effect of entrapment method. Silymarin (SM) which is a poorly water soluble drug, could be incorporated into the inner core of the micelles. The 20% SM-loaded BSCS micelles prepared via various entrapment methods including dialysis, evaporation and sonication, dropping, cosolvent evaporation exhibited different characteristics in term of entrapment efficiency, loading capacity and particle size. The 20% SM-loaded BSCS micelles properties are shown in Table 4.1. The SM-loaded micelles prepared by the dialysis, dropping and cosolvent evaporation method had smaller particle size than those prepared by evaporation and sonication method. All polymeric micelles showed highly negative charges.

Table 4.1 Particle size, polydispersity index (PDI), zeta potential of 20% SM-loaded BSCS micelles prepared by different methods (n=3).

Preparation method	Particle size (nm)	PDI	Zeta potential (mV)
Dialysis	148.3 ± 5.7	0.207	-35.5 ± 5.0
Evaporation and sonication (evap-so)	357.7 ± 97.5	0.463	-40.5 ± 4.3
Dropping	129.8 ± 12.9	0.271	-35.2 ± 4.7
Cosolvent evaporation (cosol-evap)	157.3± 10.4	0.211	-33.7 ± 2.5

Because the solubility of SM in water is approximately 0.04 mg/mL [9], the use of large volume of distilled water during dialysis may lead to the dissolution and release of SM from the inner to the outer of dialysis bag. Therefore, dialysis and dropping methods were not suitable for preparing SM-loaded polymeric micelles. On

the other hand, evaporation and sonication method (evap-so) and cosolvent evaporation method (cosol-evap) presented high entrapment efficiency and loading capacity (Table 4.2). This result indicated that the formation of SM-loaded micelles occurred through hydrophobic interactions between SM and inner core of polymer [33]. These two methods were selected to develop amphiphilic chitosan derivatives loading SM with different weight ratios of SM and various types of polymers.

Table 4.2 The entrapment efficiency (%EE), loading capacity (LC) of 20% SM-loaded BSCS micelles prepared by different methods (n=3).

Preparation method	%EE	LC (μg/mL)
Dialysis	0.00	0.00
Evap-so	56.85 $\pm$ 1.36	113.70 $\pm$ 2.71
Dropping	0.00	0.00
Ccosol-evap	80.31 $\pm$ 4.71	160.62 $\pm$ 9.43

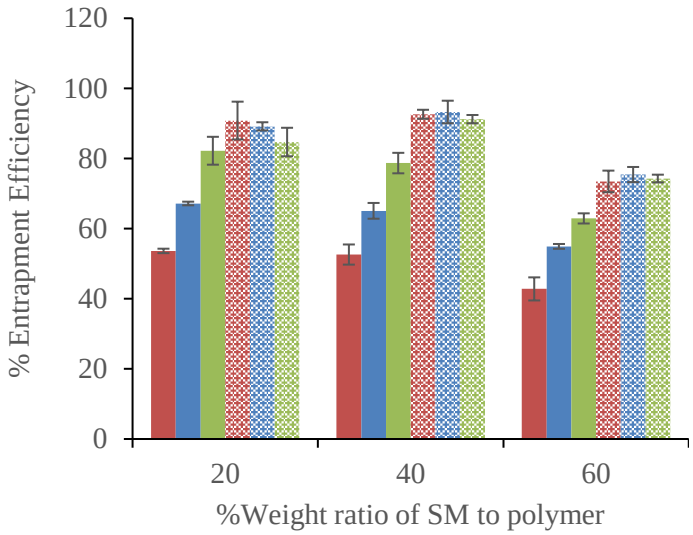
4.1.2 Effects of different weight ratios of silymarin and various types of polymers on polymeric micelles properties

As SM-loaded polymeric micelles were successfully prepared via evap-so method and cosol-evap method, three types of amphiphilic chitosan derivatives; *N*-benzyl-*N*,*O*-succinyl chitosan (BSCS); *N*-naphthyl-*N*,*O*-succinyl chitosan (NSCS); *N*-octyl-*N*-*O*-succinyl chitosan (OSCS) were used to investigate the effects of different hydrophobic moieties on the micelles properties. Different initial amounts of SM (20-60%wt to polymer) were incorporated into BSCS (SM-BSCS), NSCS (SM-NSCS) or OSCS (SM-OSCS) polymeric micelles.

4.1.2.1 Entrapment efficiency and loading capacity

The entrapment efficiency and loading capacity are shown in Figure 4.1a and 4.1b respectively.

(a)



(b)

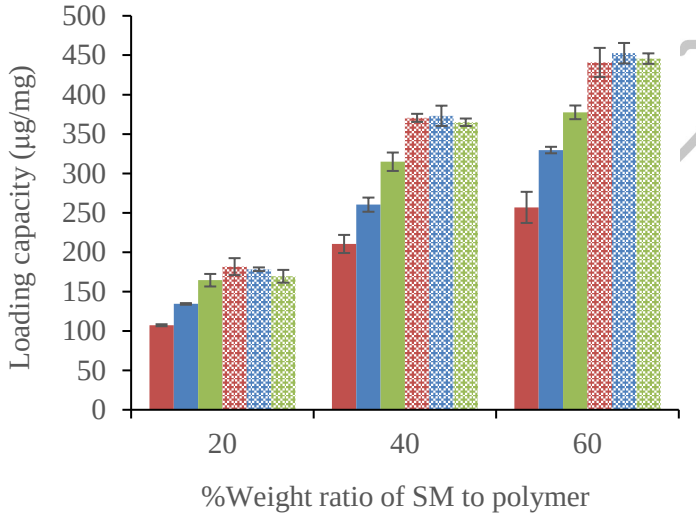


Figure 4.1 Effects of types of polymers and initial amount of SM on (a) entrapment efficiency and (b) loading capacity; (■) SM-BSCS (evap-so); (■) SM-NSCS (evap-so); (■) SM-OSCS (evap-so); (▩) SM BSCS (cosol-evap); (▩) SM- NSCS via (cosol-evap); (▩) SM- OSCS (cosol-evap). Data are presented the mean ± standard deviation of triplicate experiments.

The weight ratios of SM to polymer influenced on the entrapment efficiency and loading capacity of polymeric micelles prepared from both methods. An increase of the initial amount of SM resulted in slightly reduced the entrapment efficiency, contrast with the loading capacity.

In evap-so method, hydrophobic segments of polymers affected the entrapment efficiency and loading capacity. SM-loaded OSCS polymeric micelles exhibited the highest entrapment efficiency and loading capacity, followed by NSCS and BSCS, respectively. The results were found to be 63-82% (OSCS), 63-67% (NSCS), 43-54% (BSCS) for entrapment efficiency and 164-378 μg/mg (OSCS), 134-330 μg/mg (NSCS), 107-257 μg/mg (BSCS) for loading capacity. The loading ability of drug in polymeric micelles depends on several factors including core forming segment, hydrophobic segment length, drug concentration and compatibility between drug molecule and hydrophobic segment of polymer [29, 38]. This result revealed that long chain hydrophobic group (octyl group) had higher ability to incorporate SM compared to naphthyl and benzyl groups. These results were in agreement with previous study; effects of hydrophobic core on meloxicam-loaded polymeric micelles by using BSCS, NSCS and OSCS polymers [45].

On the other side, the incorporation of SM into BSCS, NSCS and OSCS polymeric micelles by cosol-evap method showed high entrapment efficiency and loading capacity in all polymers; SM-loaded BSCS (73-93% and 181-441 μg/mg); SM-loaded NSCS (75-93% and 178-452 μg/mg); SM-loaded OSCS showed (74-91% and 169-446 μg/mg). Since this method was modified from o/w emulsion method, the micelles forming occurred when organic solvent was evaporated after diffused into the external aqueous phase [33, 35]. In this study, drug was dissolved in organic solvent (acetone) and then dropped into blank polymeric micelles in aqueous with vigorous stirring. During the incorporation of drug into the blank polymeric micelles, acetone rapidly diffused into water [35]. After acetone completely evaporated, the small particles of drug were entrapped in the inner core of the micelles. Nevertheless, some of drug particles might be still dispersed in medium. Therefore, the variation of hydrophobic segments in polymer chain did not affect the entrapment efficiency and

loading capacity of SM-loaded BSCS, NSCS or OSCS prepared by cosol-evap method.

This result demonstrated that the initial amount of drug, the presence of hydrophobic interactions and the preparation methods play important roles in the incorporation of drug into polymeric micelles.

4.1.2.2 Particle size and zeta potential

The particle size, polydispersity index (PDI) and zeta potential of polymeric micelles measured by diffractive light scattering (DLS) are shown in Table 4.3. As a result of succinyl groups on the chitosan backbone, polymeric micelles with or without SM were highly negatively charged. The particle size of blank polymeric micelles prepared via two methods (evap-so and cosol-evap) and three amphiphilic chitosan derivatives (BSCS, NSCS, OSCS) containing different hydrophobic moieties were found to be different. The blank NSCS polymeric micelles showed the smallest size among the polymers for both preparation methods. This may result from the condensation of hydrophobic segments inside the inner core. The particles size of blank BSCS, NSCS, OSCS polymeric micelles prepared by cosol-evap method (102-155 nm) were smaller than those prepared by evap-so (160-265 nm). The incorporation of SM into BSCS, NSCS, OSCS polymeric micelles led to particle size enlargement related to the increase in weight ratio of SM to polymer. It was suggested that SM was successfully entrapped into the inner core of polymeric micelles. The average particle size of SM-loaded BSCS, NSCS and OSCS polymeric micelles were observed to be 173-327, 119-337 and 140-335 nm, respectively.

Table 4.3 Particle size, polydispersity index (PDI), zeta potential of SM-loaded BSCS, NSCS, OSCS polymeric micelles prepared by different methods (n=3).

Preparation method	Amphiphilic chitosan derivatives	SM (%wt to polymer)	Particle size (nm)	PDI	Zeta potential (mV)
Evap-so	BSCS	0	264.1 ± 53.1	0.328	-35.6 ± 4.2
		20	331.4 ± 6.2	0.342	-31.6 ± 1.0
		40	314.3 ± 33.9	0.291	-36.9 ± 1.8
		60	326.9 ± 36.3	0.334	-23.8 ± 1.5
	NSCS	0	160.9 ± 27.0	0.273	-36.4 ± 4.4
		20	214.5 ± 8.1	0.352	-33.0 ± 4.3
		40	262.9 ± 10.1	0.370	-33.5 ± 1.2
		60	336.4 ± 15.2	0.287	-30.3 ± 2.0
	OSCS	0	265.0 ± 27.9	0.304	-31.5 ± 1.0
		20	320.6 ± 19.1	0.356	-31.9 ± 0.7
		40	335.5 ± 55.4	0.345	-32.4 ± 0.8
		60	335.1 ± 38.1	0.246	-27.7 ± 1.0
Cosol-evap	BSCS	0	154.8 ± 3.3	0.282	-38.8 ± 1.6
		20	173.5 ± 3.1	0.249	-32.6 ± 6.8
		40	177.3 ± 14.3	0.256	-28.4 ± 1.3
		60	186.6 ± 24.5	0.229	-31.3 ± 1.6
	NSCS	0	102.5 ± 16.1	0.612	-30.5 ± 1.0
		20	119.9 ± 1.5	0.094	-29.3 ± 2.2
		40	153.1 ± 25.7	0.216	-27.8 ± 0.5
		60	137.0 ± 12.3	0.175	-28.3 ± 4.6
	OSCS	0	128.3 ± 12.0	0.318	-28.7 ± 0.1
		20	140.2 ± 2.0	0.271	-29.2 ± 1.4
		40	176.4 ± 48.2	0.249	-25.1 ± 5.4
		60	324.2 ± 96.2	0.289	-27.0 ± 3.2

4.1.2.3 Morphology and structure stability

The morphology of SM-loaded polymeric micelles observed by transmission electron microscope (TEM) are shown in Figure 4.2. The spherical shape confirmed the core-shell structure of SM-loaded polymeric micelles [28-30].

(a) (b)

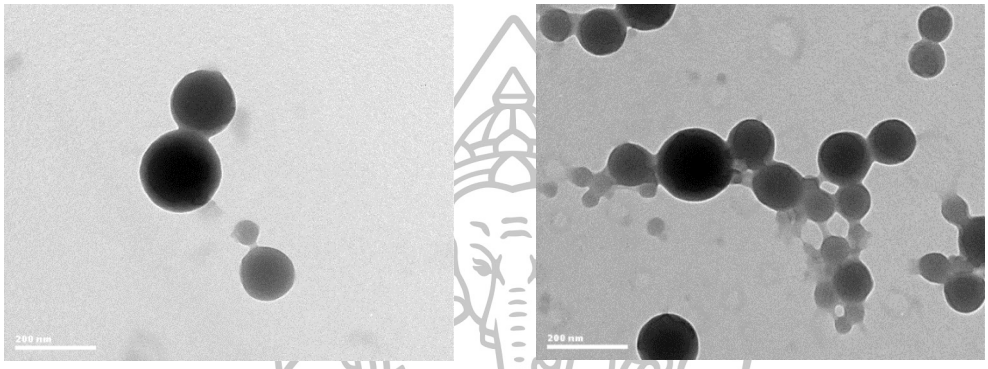


Figure 4.2 The TEM images for SM-BSCS prepared via (a) evap-so and (b) cosol-evap.

The structure stability of SM-loaded BSCS, NSCS and OSCS prepared by cosol-evap method and evap-so method with different ratios of SM to polymers were assessed by gel permeation chromatography (GPC). The polymeric micelles were passed through a GPC column under aqueous flow condition [31-32, 39]. The polymeric micelles were detected by RI detector meanwhile the drug incorporated in the inner core of polymeric micelles was detected by UV detector. Both peaks have to be eluted at the same retention time [46]. The ratios of peak area of drug found/concentration of drug injected [SM] are shown in Figure 4.3. This result represented the stability of the micelles against dissociation in the column and the strength of hydrophobic interaction between drug and polymer [32]. The high value of the ratio implying high drug content was loaded into the polymeric micelles. From these results, an increase in weight ratios of SM to polymers trended to decrease the structure stability in all BSCS, NSCS and OSCS polymeric micelles prepared by both methods. Initial amount of SM with 20%wt to polymer loading in amphiphilic

chitosan derivatives showed highest structure stability. Although, SM-loaded polymeric micelles prepared by evap-so showed higher peak area/[SM] and the peaks were sharper than the micelles prepared by cosol-evap method. It was probable that a high drug content made the micelles to easily dissociate. Moreover, the hydrophobic interaction of different hydrophobic segments of amphiphilic chitosan polymers appeared to be different. SM-loaded NSCS micelles showed the highest peak area which indicated the highest structure stability, followed by BSCS and OSCS, respectively. This result suggested that SM has stronger interactions with naphthyl group than benzyl and octyl groups.

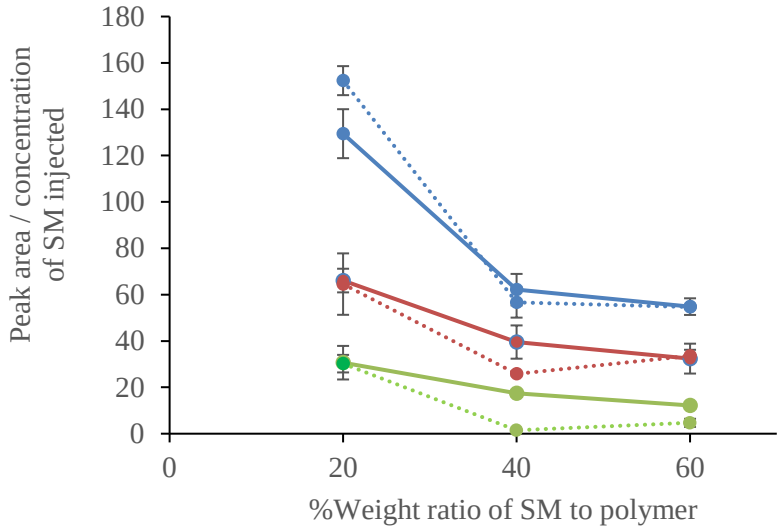


Figure 4.3 Effects of various types of polymers and initial amount of SM loading on structure stability; (-●-) SM-BSCS (evap-so); (-●-) SM-NSCS (evap-so); (-●-) SM-OSCS (evap-so); (···●···) SM-BSCS (cosol-evap); (···●···) SM-NSCS (cosol-evap); (···●···) SM-OSCS (cosol-evap). Data are presented the mean ± standard deviation of triplicate experiments.

4.2 *In vitro* drug release

60% initial of SM-loaded micelles (BSCS, NSCS and OSCS) exhibited high entrapment. The morphology and structure stability of SM-loaded micelles prepared by evap-so method were more stable than cosol-evap method. Consequently, SM-loaded BSCS, NSCS and OSCS micelles prepared by evap-so method were selected for investigation of the release behavior.

4.2.1 pH 1.2 changed to 6.8

Since amphiphilic chitosan derivatives (BSCS, NSCS and OSCS) is a pH sensitive polymers, SM release were studied in the medium complied with gastrointestinal (GI) pH. At first 2 h, 0.1 N HCl with NaCl medium (pH 1.2) corresponding to gastric fluid (SGF) was used. Then the pH was changed to 6.8 with trisodium phosphate as the intestinal fluid (SIF). The drug release from BSCS, NSCS, OSCS polymeric micelles and free SM (SM suspension) was performed using dialysis bag method. The result is shown in Figure 4.4. In pH 1.2 medium for 2 h, less than 25% of SM were released from SM-loaded BSCS, NSCS or OSCS micelles, but these amounts were higher than free drug (approximately 6%). When the pH was changed to 6.8, the pertcentage cumulative release of SM from all SM-loaded polymeric micelles was considerably increased due to the dissociation of the polymeric micelles. This indicated that BSCS, NSCS and OSCS micelles was pH-responsive polymeric micelles. The succinyl moieties in polymer chains containing carboxyl groups were ionized at the pH above pK_a (approximately 4.2) and induced the dissociation of polymeric micelles [44-45]. The morphology change in various pH was observed in the previous study [43-45]. In acidic medium (pH 1.2), BSCS, NSCS and OSCS micelles exhibited small and condense particles. In pH 6.8 medium, all polymeric micelles swelled leading to drug release [43-45]. In case of free SM, slow release of drug in both medium was observed. The amount of drug release from SM-loaded all polymeric micelles in SIF medium was significantly higher ($p<0.05$) than free drug due to poor solubility of SM in aqueous medium. The incorporation of SM into polymeric micelles successfully increased the solubility of SM and increased the drug release in GI medium. Moreover, among SM-loaded polymeric micelles, OSCS micelles seemed to give higher drug release compared with BSCS and NSCS micelles because of its

lower structure stability. Nevertheless, it was not significantly different. The polymeric micelles dissociation inducing drug release depends on structure stability, pH, hydrophobic chain length of polymer, cohesion of micelles core, presence of salts in medium, etc. [31-32]. This result showed that polymeric micelles made from amphiphilic chitosan derivatives (BSCS, NSCS, OSCS) can act as potential carriers for SM oral administration.

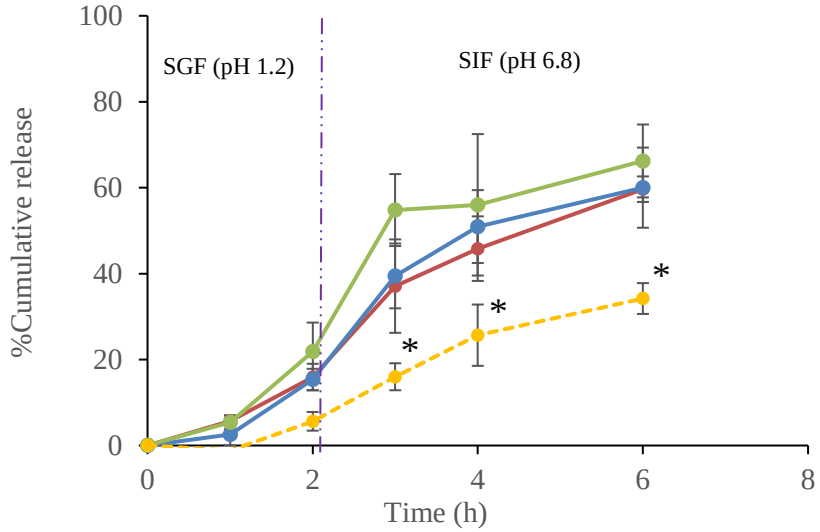


Figure 4.4 Release profile of (-●-) SM-BSCS; (-●-) SM-NSCS; (-●-) SM-OSCS; (···●···) free SM (SM suspension) in pH 1.2 for 2 h then changed to pH 6.8 for 4 h. Data are presented the mean ± standard deviation of triplicate experiments. * Statistically significant ($p<0.05$).

4.2.2 pH 7.4

The release behavior of SM-loaded BSCS, NSCS, OSCS micelles and free drug in phosphate buffer saline (PBS) medium (pH 7.4) was investigated by dialysis bag method. The release rate of all samples rapidly increased in first 2 h, then slightly decreased. After 4 h, the percentage of cumulative release reached the highest value (Figure 4.5). There was no significant difference among the release profiles of SM-

loaded BSCS, NSCS, OSCS polymeric micelles. The percentage cumulative release of SM-loaded BSCS, NSCS, OSCS micelles was found to be ~ 87%, ~ 81%, ~86%, respectively. Nevertheless, the release profile of free SM (SM suspension) was significantly lower ($p<0.05$) than SM-loaded polymeric micelles, it was found to be ~ 58%. It is indicated that amphiphilic chitosan derivatives could enhance SM release behavior due to the higher solubility of SM-loaded polymeric micelles. Additionally, succinyl moieties in amphiphilic chitosan derivatives polymer chains can be ionized and swollen in the medium of pH greater than pK_a, leading to the dissociation of the polymeric micelles [43]. Consequently, all polymeric micelles dissociated in PBS pH 7.4 and greatly released SM from the polymeric micelles.

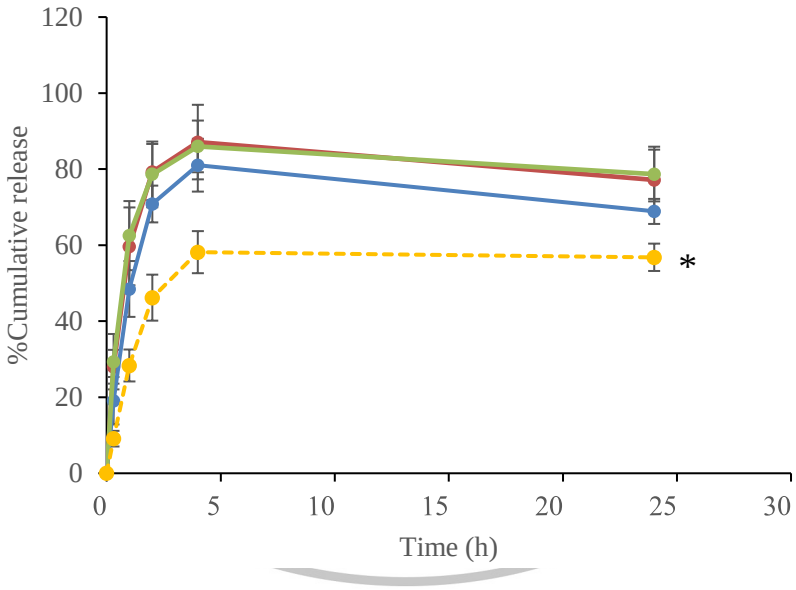


Figure 4.5 The release profile of (-●-) SM-BSCS; (-●-) SM-NSCS; (-●-) SM-OSCS; (···●···) free SM (SM suspension) in pH 7.4. Data are presented the mean ± standard deviation of triplicate experiments. * Statistically significant ($p<0.05$).

From SM release behavior in both experiments, SM free drug release profile seemed to depend on the pH of medium. However, the greater percentage cumulative release of SM-loaded polymeric micelles compared to free drug was observed. Different release profile in various pH medium indicated that amphiphilic chitosan derivatives were pH sensitive polymers which can act as potential drug carriers for oral administration and parenteral administration.

4.3 Permeation study

Since SM is generally given orally and poor bioavailability has been noticed [58], permeation study should be investigated. In oral absorption process, solubilization of drug within gastrointestinal tract might be a critical step affecting oral bioavailability [47]. The oral administration is considered as the most convenient route because of painless self-medication [37]. The varietal pH occurs in various part of GI tract including pH 1.2 in empty stomach, pH 5-7 in small intestine and pH 6-7.5 in colon [37]. The small intestine is determined as a major site of drug absorption [37]. In this study, the permeation of SM across the porcine intestinal membrane was examined. 2% Sodium lauryl sulfate (SLS) in PBS was used as receiver medium according with the dissolution medium of SM in USP [60]. The cumulative of SM/area in each sampling time is shown in Figure 4.6. At first 30 minutes, dissolved SM from free SM (SM suspension) rapidly permeated through porcine intestinal membrane while dissolved SM from SM-loaded polymeric micelles was not detected. It was resulted from that SM-loaded polymeric micelles needed more time to dissociate and permeated through the intestinal membrane. However, the rate of SM permeation from SM-loaded polymeric micelles after 1 h trended to be faster than free SM due to the high solubility of SM-loaded polymeric micelles compared to free SM. The cumulative of SM permeated from SM-BSCS and SM-OSCS through porcine intestinal membrane (at 1-4 h) was significantly higher than that of free SM.

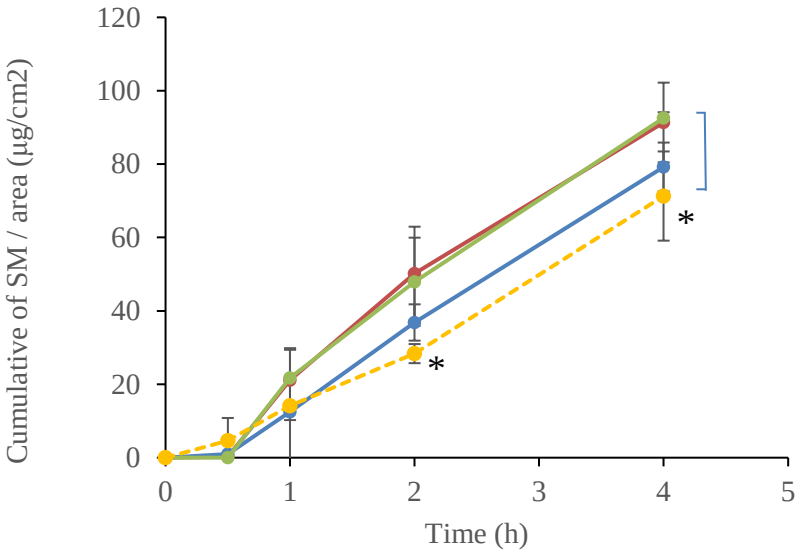


Figure 4.6 The permeation profile of (-●-) SM-BSCS; (-●-) SM-NSCS; (-●-) SM-OSCS; (···●···) free SM (SM suspension) in PBS pH 7.4 with 2% SLS. Data are presented the mean ± standard deviation of triplicate experiments. * Statistically significant ($p<0.05$).

The fluxes of SM-loaded polymeric micelles and free SM are demonstrated in Figure 4.7. The fluxes of SM-loaded BSCS and OSCS polymeric micelles were higher than free SM because only dissolved form of SM could be absorbed. After polymeric micelles completely dissociated, the dissolved drug loading in the inner core of polymeric micelles was promptly absorbed through intestinal membrane in contrast to free SM which was slowly dissolved. Additionally, the flux and amount of SM entrapped in NSCS polymeric micelles was lower than those in BSCS and OSCS due to the higher structure stability in accordance with GPC study. This results suggested that the amphiphilic chitosan derivatives (BSCS, NSCS, OSCS) have a potential to use as SM carriers for improving drug solubility. In addition, improving bioavailability by increase rate of permeation due to high solubility was considered.

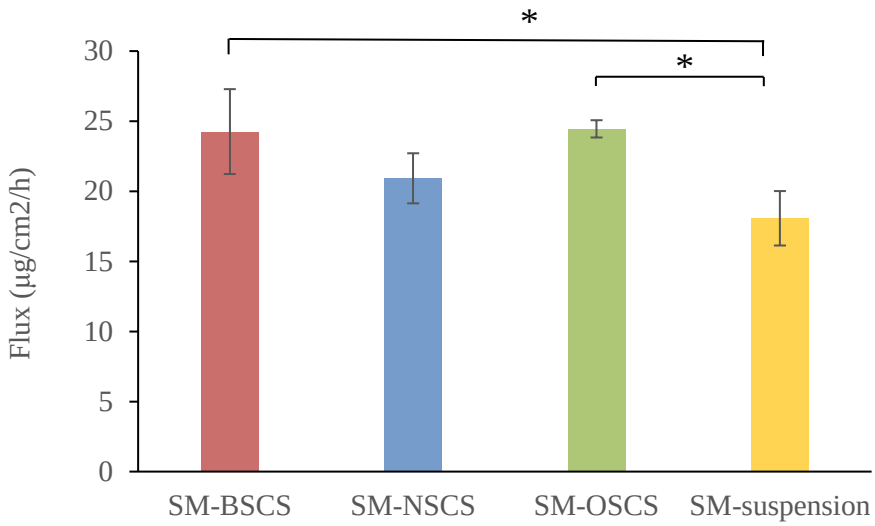


Figure 4.7 The fluxes of SM permeated across porcine intestinal membrane for SM-loaded BSCS, NSCS, OSCS polymeric micelles and free SM (SM suspension); (■) SM-BSCS; (■) SM-NSCS; (■) SM-OSCS; (■) free SM (SM suspension). Data are presented the mean ± standard deviation of triplicate experiments. * Statistically significant ($p<0.05$).

4.4 Cytotoxicity

The cytotoxicity of SM, blank polymeric micelles and SM-loaded polymeric micelles in both head and neck squamous cell carcinoma (HN22 cells) and human renal proximal tubular epithelial cells (RPTEC/TERT1) was assessed by MTT test. Relative cell viability (%) was measured and compared with non-treated cells (control).

4.4.1. Head and neck cancer cells (HN22)

Since cisplatin has been used to treat head and neck cancer in patients [1-4], in this study, HN22 cells were used for cisplatin treatment. The cytotoxicity of SM, blank polymeric micelles and SM-loaded polymeric micelles in HN22 cells was evaluated.

4.4.1.1. Free SM

The percentage of cell viability of free SM on HN22 cells evaluated by MTT assay is illustrated in Figure 4.8(a). The IC_{50} value calculated by non-linear regression model is shown in Figure 4.8(b). The IC_{50} value of SM was found to be 142.1 µg/mL.

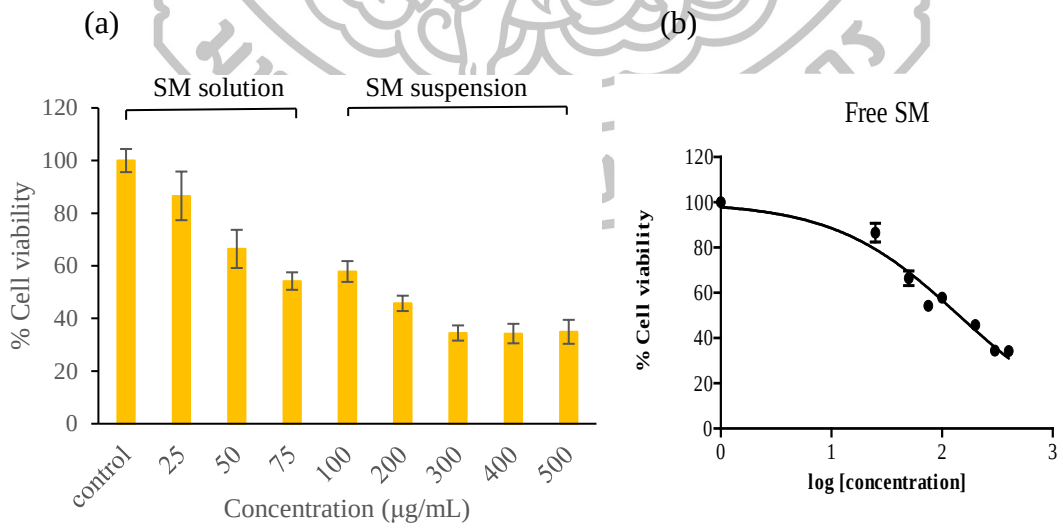


Figure 4.8 Cytotoxicity of SM in HN22 cells. (a) % cell viability compare with non-treated cells (control); (b) determination of the IC_{50} by non-linear regression. Each value represents the mean ± standard deviation.

4.4.1.2. Blank polymeric micelles

The cytotoxicity of blank micelles (BSCS, NSCS, OSCS) on HN22 evaluated by MTT assay is illustrated in Figure 4.9. The % cell viability was determined and compared with non-treat cells in each experiment. The IC_{50} value of blank BSCS, NSCS, OSCS polymeric micelles were 1,040.0, 786.1 and 445.0 μg/mL, respectively. The results indicated that OSCS polymeric micelles had the highest toxicity against HN22 cells.

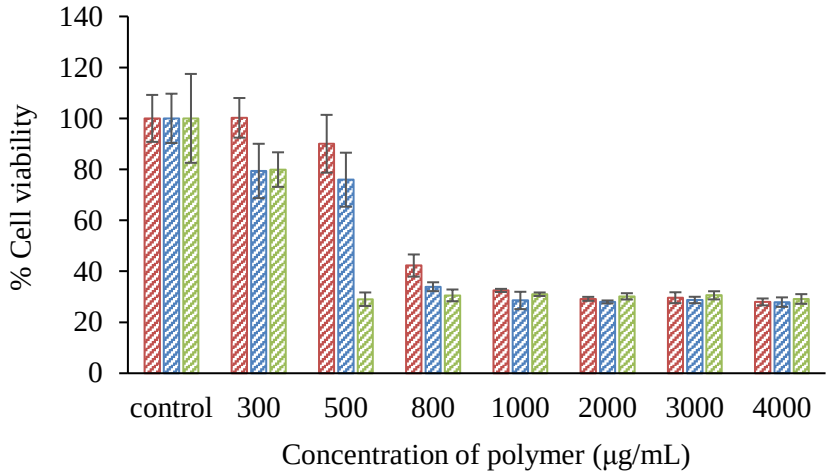


Figure 4.9 The % cell viability of blank polymeric micelles (▨)BSCS; (▨)NSCS; (▨) OSCS in HN22 cells. Each value represents the mean ± standard deviation.

4.4.1.3. SM-loaded polymeric micelles

The cytotoxicity of SM-loaded polymeric micelles on HN22 determined by MTT test is shown in Figure 4.10. It was found that SM-OSCS polymeric micelles was the most toxic, followed by SM-NSCS and SM-BSCS polymeric micelles, respectively. The non-linear regression model of percent cell viability is shown in Figure 4.10(b). The IC_{50} value of SM-loaded micelles was calculated as the concentration of SM in micelles. The IC_{50} values of SM-BSCS, SM-NSCS and SM-OSCS were 347.6, 251.6 and 128.2 μg/mL, respectively. In comparison, the free SM had a higher toxicity (IC_{50}~142.1 μg/mL) than SM-BSCS (IC_{50}~347.6 μg/mL) and SM-NSCS (IC_{50}~251.6 μg/mL), but it had a lower cytotoxicity than SM-OSCS (IC_{50}~128.2 μg/mL). From the

results, it was suggested that the cytotoxicity of SM-loaded micelles depends on the concentration of SM entrapped in the inner core, concentration of polymers and type of polymers. OSCS polymer showed the highest toxicity in both blank polymeric micelles and SM-loaded micelles.

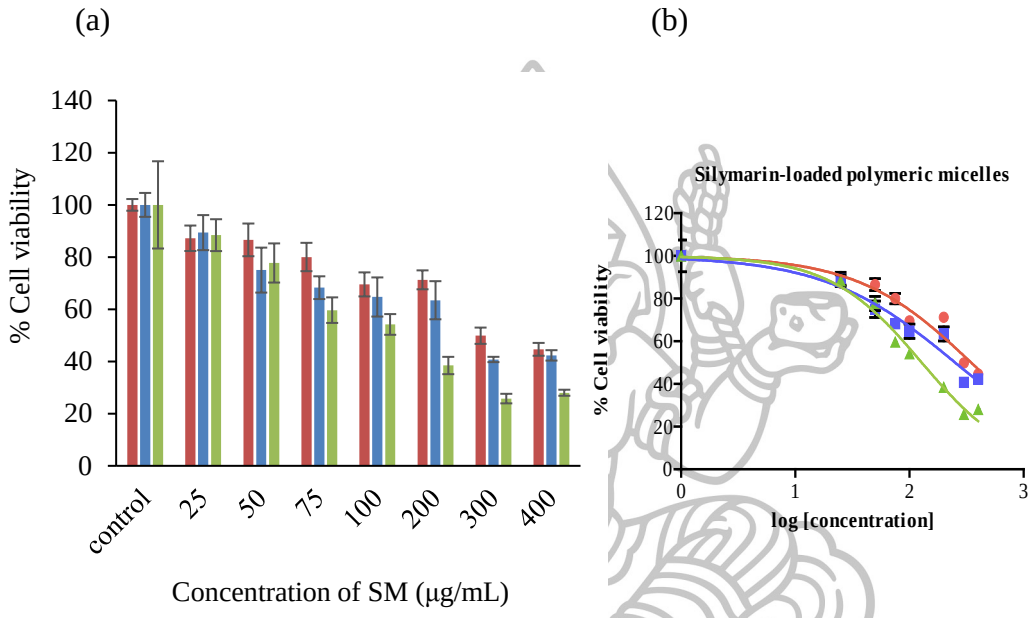


Figure 4.10 Cytotoxicity of SM-loaded micelles in HN22 cells (a) % cell viability compare with non-treated cells (control); (■) SM-BSCS; (■) SM-NSCS; (■) SM- OSCS; (b) determination of the IC_{50} by non-linear regression. (●) SM-BSCS; (■) SM- NSCS; (▲) SM- OSCS. Each value represents the mean ± standard deviation.

4.4.2. Renal cells (RPTEC/TERT1)

The RPTEC/TERT1 cells are normal proximal tubular epithelial cells which represent the morphology and functional properties of human kidney [47-48]. The cytotoxicities of free SM, blank polymeric micelles and SM-loaded polymeric micelles in RPTEC/TERT1 cells were evaluated by MTT assay to investigate the responses of the normal renal cells to SM and the polymers.

4.4.2.1. Free SM

Previous studies suggested that SM is a low toxic agent and has a renoprotective effects [17, 19]. In this study, RPTEC/TERT1 cells were used as a model to demonstrated the toxic of SM to normal renal cells. The cytotoxicity of free SM was investigated with various concentration of SM (0-200 µg/mL). The cell viability was considerably decreased when the concentration of SM increased to 200 µg/mL (Figure 4.11). The result showed that the IC_{50} value was 146.2 µg/mL confirming the low toxicity of SM.

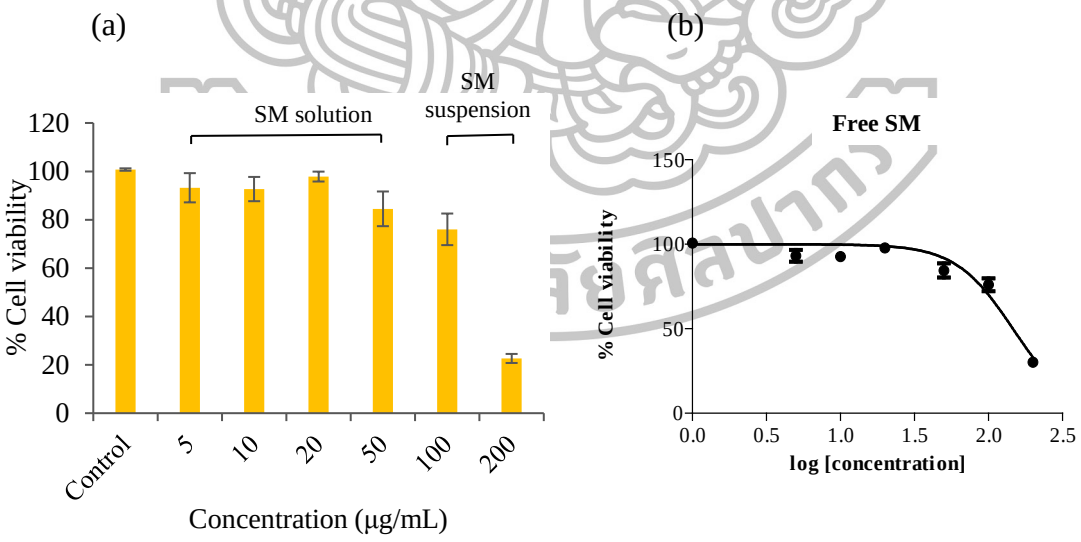


Figure 4.11 Cytotoxicity of free SM in RPTEC/TERT1 cells. Each value represents the mean ± standard deviation.

4.4.2.2. Blank polymeric micelles

The % cell viability determined by MTT assay is shown in Figure 4.12. The cytotoxicity of BSCS, NSCS, OSCS blank polymeric micelles were found to be different. For blank BSCS polymeric micelles, the % cell viability decreased when the concentration of polymeric micelles increased. Conversely, the percent cell viability of blank NSCS and OSCS polymeric micelles increased at the concentration of 100 – 1,000 µg/mL, compared with non-treat cells, whereas the percent cell viability was extremely decreased when the concentration reached 2,000 µg/mL for blank NSCS and 3,000 µg/mL for blank OSCS micelles. Among blank micelles, BSCS micelles were the lowest toxic and did not interfere with the cell growth.

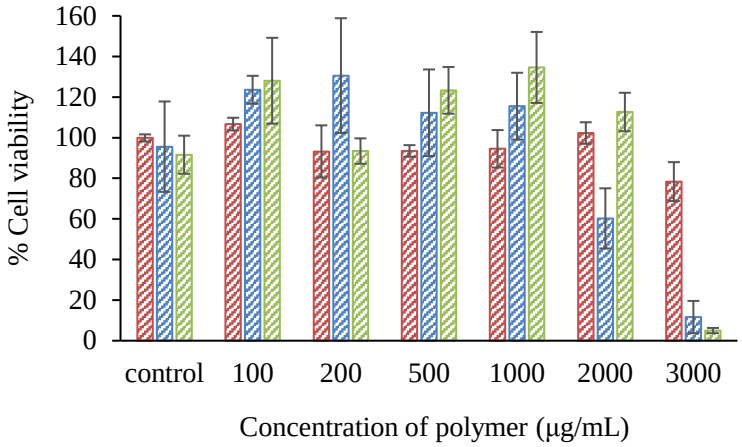


Figure 4.12 The % cell viability of blank polymeric micelles ( )BSCS; ( )NSCS; ( ) OSCS in RPTEC/TERT1 cells. Each value represents the mean ± S.D. (n=4)

4.4.2.3. SM-loaded polymeric micelles

The effects of SM-loaded polymeric micelles on the RPTEC/TERT1 cells were evaluated by MTT assay. The cytotoxicity of SM-BSCS, SM-NSCS and SM-OSCS is demonstrated in Figure 4.13. The % relative cell viability via various concentration of SM loading in polymeric micelles is shown in Figure 4.13(a). The non-linear regression model of % cell viability and log[SM concentration] is shown in Figure 4.13(b). The % cell viability of less than 30% occurred when SM concentration was above 100 µg/mL in all polymeric micelles. At the low concentration, SM-OSCS increased cell viability compared to non-treated cells which was similar to blank OSCS polymeric micelles. The % cell viability of SM-loaded polymeric micelles were different among the polymers but were not statistically significant. The IC_{50} values of SM-BSCS, SM-NSCS and SM-OSCS were 153.2, 133.4 and 180.3 µg/mL, respectively. The concentration of SM loading in polymeric micelles and type of polymers influenced on the cytotoxicity of SM-loaded polymeric micelles in RPTEC/TERT1 cells. An increase of SM concentration led to the decrease of cell viability.

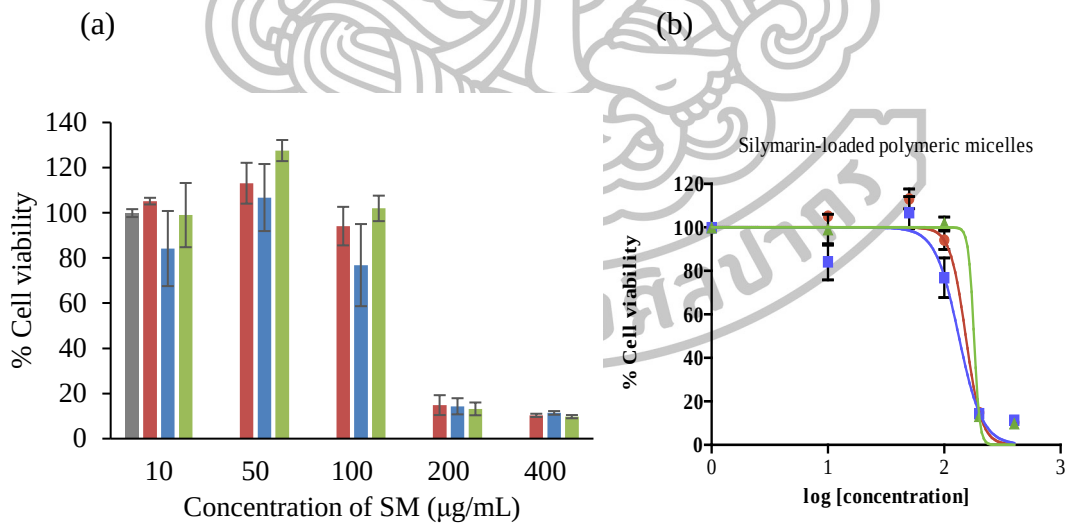


Figure 4.13 Cytotoxicity of SM-loaded polymeric micelles in RPTEC/TERT1 cells (a) % cell viability compare with non-treated cells; (■) control; (■) SM-BSCS; (■) SM- NSCS; (■) SM- OSCS (b) determination of the IC_{50} by non-linear regression. (●) SM-BSCS; (■) SM- NSCS; (▲) SM- OSCS. Each value represents the mean ± standard deviation.

4.5 Effects of free SM and SM-loaded micelles on head and neck cancer cells during treatment with cisplatin

Since head and neck cancer is one of the targets of cisplatin treatment, the killing effects of cisplatin treatment with SM and SM-loaded polymeric micelles on HN22 cells were determined.

4.5.1. Cisplatin cytotoxicity

The % cell viability of cisplatin and the IC_{50} values of cisplatin are illustrated in Figure 4.14(a) and 4.14(b), respectively. Low concentration of cisplatin could induce cell death. The IC_{50} value of cisplatin was approximately 8.7 μg/mL (~ 30 μM). The cytotoxicity of cisplatin in HN22 cells depended on the concentration of cisplatin.

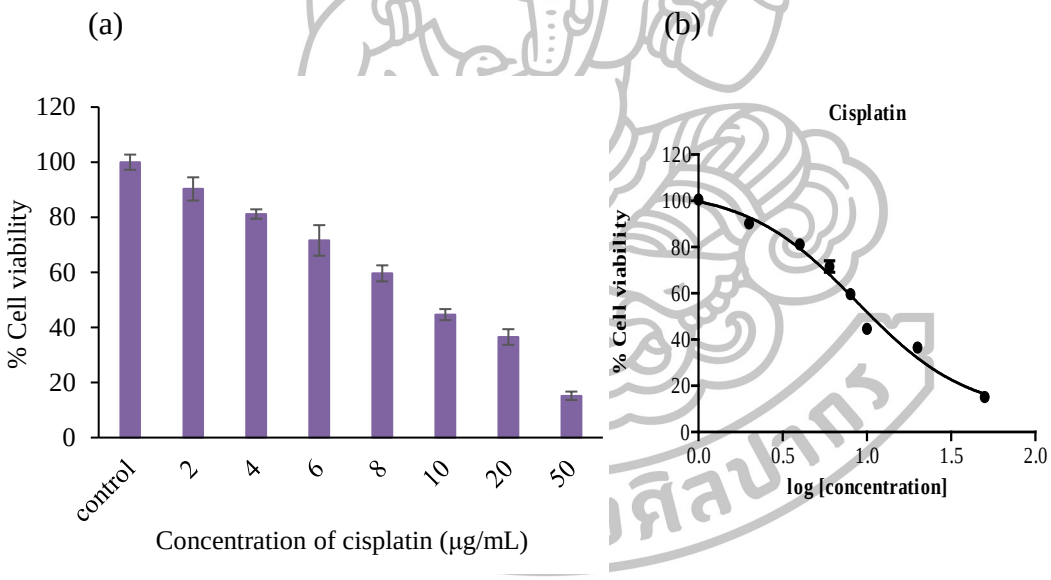


Figure 4.14 Cytotoxicity of cisplatin in HN22 cells (a) % cell viability compare with non-treated cells (control); (b) determination of the IC_{50} by non-linear regression. Each value represents the mean ± standard deviation.

4.5.2. Effects of free SM and SM-loaded micelles pre-treatment

The HN22 cells were pre-treated with various concentration of free SM and SM-loaded polymeric micelles for 1 h, then cisplatin was added to the final concentration of 30 μM. After 24 h, the % cell viability was observed by MTT test. It was revealed that pre-treatment of free SM or SM-loaded polymeric micelles (BSCS, NSCS, OSCS) did not increase the % cell viability compared with the cisplatin treatment without SM (Figure 4.15).

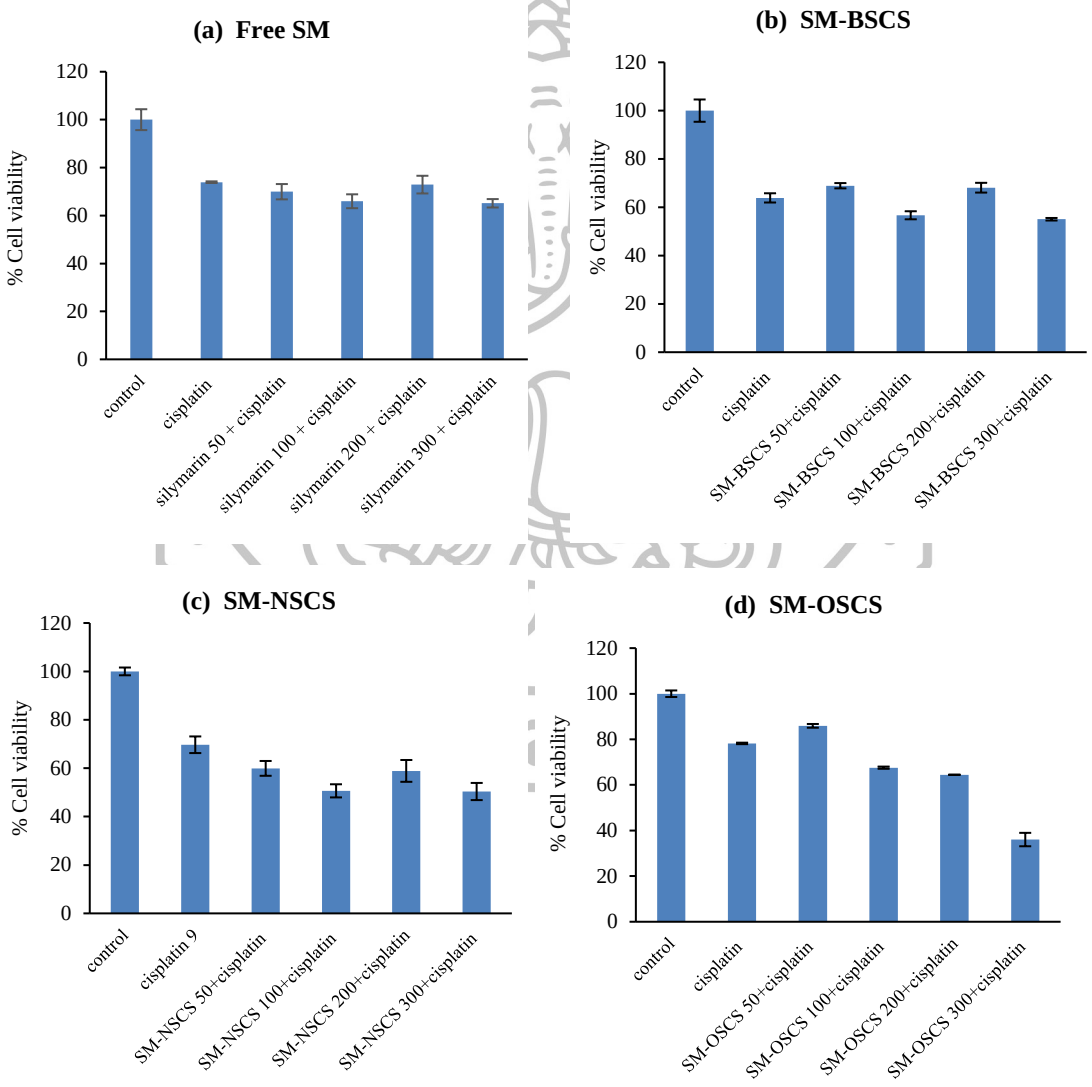


Figure 4.15 Pre-treatment effects of (a) free SM; (b) SM-BSCS; (c) SM-NSCS; (d) SM-OSCS during treatment with cisplatin. Each value represents the mean ± standard deviation.

4.5.3 Effects of free SM and SM-loaded micelles co-treatment

The killing effects of co-treatment of various concentration of free SM and SM-loaded polymeric micelles with cisplatin 30 μM on HN22 cells were studied by MTT assay. The results were similar to the pre-treatment. The % cell viability during treatment with cisplatin was not affected by SM and SM-loaded polymeric micelles (Figure 4.16).

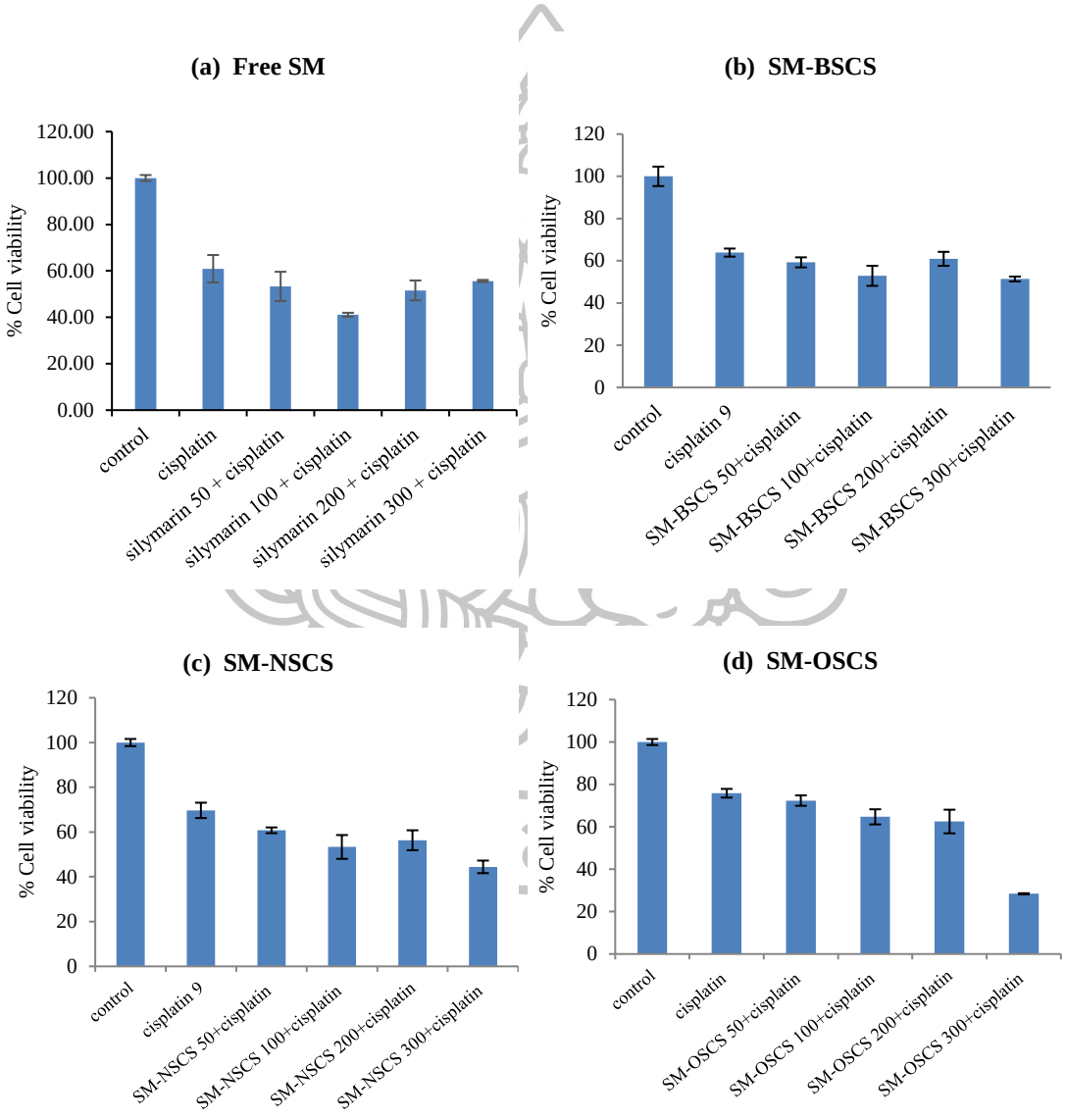


Figure 4.16 Co-treatment effects of (a) free SM; (b) SM-BSCS; (c) SM-NSCS; (d) SM-OSCS during treatment with cisplatin. Each value represents the mean ± standard deviation.

It was suggested that the efficacy of cisplatin in HN22 cancer cells treatment was not reduced by pre- or co- treatment with free SM and SM-loaded polymeric micelles. Furthermore, the high concentration of SM-OSCS polymeric micelles (300 μg/mL) could enhance the killing effects of cisplatin in HN22 cells. This indicated that SM can be used in combination with cisplatin since it did not reduce cisplatin efficacy.

4.6 Effects of free SM and SM-loaded micelles on renal cells against cisplatin-induced nephrotoxicity

Since renal toxicity is the most serious side effect which usually occurs in patient treated with cisplatin [3], several studies have been investigated to discover effective renoprotective agents. SM is commonly known as a hepatoprotective agent whereas recently studies reported that SM exhibits protective effect against drug-induced nephrotoxicity including cisplatin [19]. In this study, SM-loaded polymeric micelles were developed to improve SM solubility. The protective effects of SM-loaded polymeric micelles against cisplatin-induced nephrotoxicity in human proximal tubular cells were evaluated compared with free SM.

The principal accumulation site of cisplatin is proximal tubular cells [4]. Cultured human kidney cell lines namely human kidney 2 (HK-2) and human renal proximal tubular epithelial cells (RPTEC/TERT1) were used as model systems to investigate the drug induced-nephrotoxicity [49-50], including the study of responses of renal cell to benzo[a]pyrene and cadmium were examined in RPTEC/TERT1 cell line [48], the transcriptomics, proteomics, metabolomics and biokinetics of sub-cytotoxic concentration of cisplatin were determined in RPTEC/TERT1 cells for up to 14 days [3], the impact of SM in the inhibition of cisplatin induced renal cell death was observed in HK-2 cells [23], and the reduction effect of fenofibrate on cisplatin-induced renal proximal tubular cells apoptosis was performed in HK-2 cells and LLC-PK1 cells [51]. Both HK-2 cells and RPTEC/TERT1 cells represent the function of the proximal tubular cells. However, HK-2 cells are lack of particular transport channels established in renal tissue because they were immortalized by a recombinant retrovirus containing the human papilloma virus E6/E7 [49-50]. While

RPTEC/TERT1 cells were immortalized by the human telomerase reverse transcriptase (hTERT) which can maintain the proximal tubule functions and expression [49-50]. Thus, it is suggested that RPTEC/TERT1 cells are appropriate for nephrotoxicity test [50].

In this study, RPTEC/TERT1 cells were selected for the investigation of the protective effects of SM-loaded polymeric micelles against cisplatin-induced nephrotoxicity. The cytotoxicity of cisplatin in RPTEC/TERT1 and HK-2 cells was performed in previous study in which the IC_{50} value of cisplatin in RPTEC/TERT1 and HK-2 cells were 451.7 and 197.1 μM, respectively with incubation time of 24 h [50]. Another previous study showed that the IC_{50} value of cisplatin in LLC-PK1 and HK-2 cells was 50 and 100 μM with incubation time of 24 h [51]. Additionally, the geometric mean of the IC_{50} value of cisplatin in pan-cancer was found to be 17.6 μM [52,59]. The IC_{50} value of cisplatin in 80% of screened cancer cell (893) lines was lower than 50 μM [52, 59]. Therefore, 50 μM of cisplatin was used in this study to determine the renoprotective effects of free SM and SM-loaded polymeric micelles.

4.6.1. MTT assay

4.6.1.1. Free SM

The preventive effects of SM were measured by MTT assay and the result is illustrated in Figure 4.17. RPTEC/TERT1 cells were pre-treated with various concentrations of SM (10 – 100 μg/mL) for 1 h, then cisplatin was added to the final concentration of 50 μM. After 72 h, the percentage of cell viability was determined. Free SM exhibited low toxicity in the concentration range of 10 – 100 μg/mL, giving the cell viability of more than 70%. Pre-treatment with free SM 50 and 100 μg/mL significantly increased cell viability ($p < 0.05$) compared to cisplatin without pre-treatment. The % cell viability increased from 30% to 65 and 90% for SM 50 and 100 μg/mL, respectively. This result indicated that SM has a protective effect against cisplatin-induced cell death.

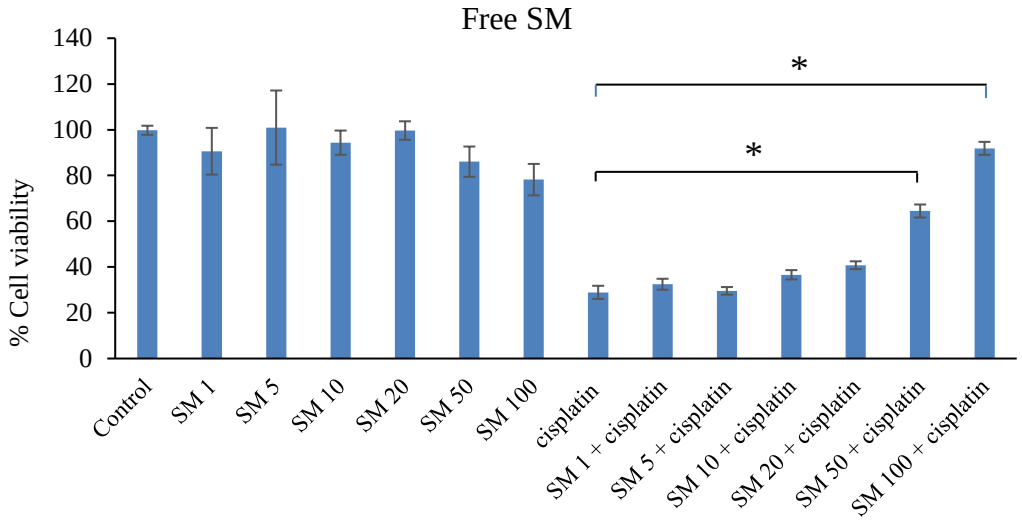


Figure 4.17 The protective effects of free SM pre-treatment followed by cisplatin treatment for 72 h measuring by MTT assay. Each value represents the mean ± standard deviation. * Statistically significant ($p<0.05$).

4.6.1.2. SM-loaded BSCS micelles

The concentrations of SM (10 – 200 µg/mL) in SM-BSCS were used for RPTEC/TERT1 cells pre-treatment. The cell viability determined by MTT assay is shown in Figure 4.18. This experiment was investigated as the same procedure as that of free SM. High concentration of 200 µg/mL exhibited great cytotoxicity, but the concentration in range of 10 – 100 µg/mL showed low cytotoxicity. The preventive effects of SM-BSCS was similar to that of the free drug. The cell viability in pre-treatment with 50 and 100 µg/mL SM-BSCS significantly increased ($p < 0.05$) compared with cisplatin without pre-treatment. The % cell viability increased from 55% to 80 and 95% for SM 50 and 100 µg/mL, respectively. It was illustrated that SM-BSCS could prevent cisplatin-induced cell death.

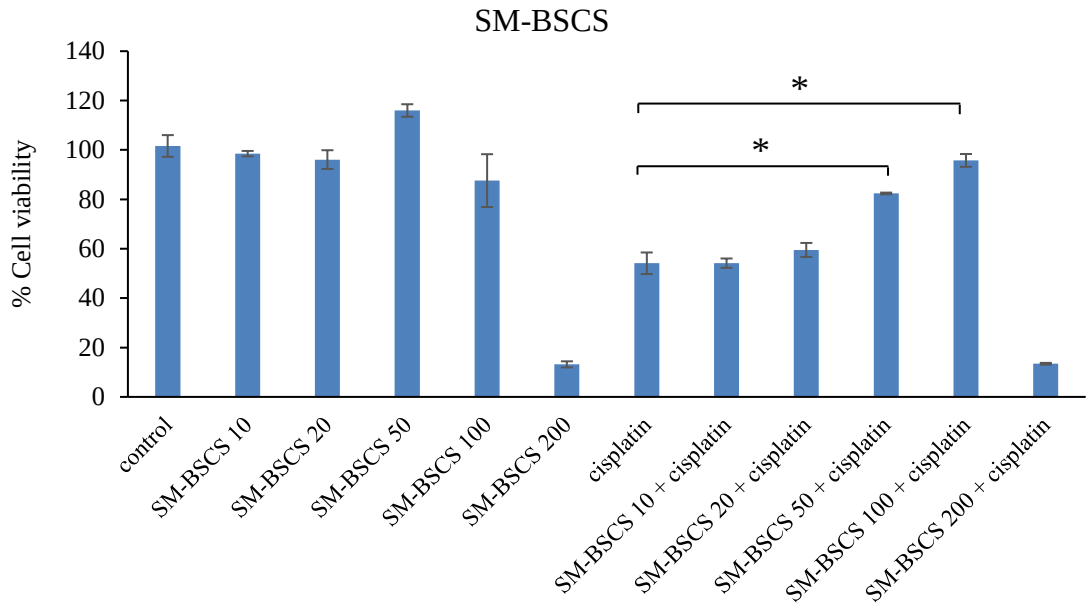


Figure 4.18 The protective effects of SM-BSCS pre-treatment followed by cisplatin treatment for 72 h measuring by MTT assay. Each value represents the mean ± standard deviation. * Statistically significant ($p<0.05$).

4.6.2. Apoptosis and necrosis detection

Since cisplatin can induce both apoptosis and necrosis, the histopathological characteristics of RPTEC/TERT1 cells were monitored. In case of apoptosis, the characterizations of morphological changes are chromatin condensation, cell membrane blebbing, nuclear fragmentation, apoptosis body and cell shrinkage [53-54]. Apoptosis is a programmed cell death which normally appear in human body [54-55]. In contrast, necrosis is stimulated by unusual conditions such as inflammation, heat, osmotic shock, ATP depletion, ischemia, cell stress, etc. [53]. Necrosis is characterized by rapid cycloplasmic swelling, plasma membrane rupture and organelles breakdown [53-54]. Several methods have been developed to evaluate apoptosis and necrosis, including cell morphology analysis, cell surface markers analysis, intracellular markers analysis, extracellular markers analysis and cell-cell interaction analysis [53]. Cell morphology analysis techniques using light microscope, fluorescence microscope, electron microscope and phase contrast microscope have

been used for detection of apoptosis and necrosis in several studies [53, 55-56]. For fluorescence microscopy method, fluorescent dyes (Hoechst 33342, DAPI, propidium iodide) are used for cell staining [55].

In this study, co-staining of Hoechst 33342 (cell membrane permeable dye) and propidium iodide (PI) (cell membrane impermeable dye) in RPTEC/TERT1 cells was conducted to detect apoptosis and necrosis cell death. The morphology of RPTEC/TERT1 after treatment with different regimen was observed under light microscope and fluorescence microscope (Figure 4.19). The non-treated cells (control) presented high density of cells and the cell structure was oval shape. The fluorescence image showed the normal distribution of less bright Hoechst dye which represented the normal chromatin in the cells (Figure 4.19a). Conversely, extremely morphological change in the cells treated with cisplatin 50 μM was occurred (Figure 4.19b). The shrinkage cells and bright blue fluorescence which stand for chromatin condensation, showed apoptosis cell death [56-57]. While the cycloplasmic swelling, plasma membrane rupture and red spots of PI represented cell necrosis [56]. Moreover, death cells detached from the cell culture plate were observed [56].

The SM at concentration of 50 and 100 μg/mL from free SM and SM-loaded BSCS polymeric micelles were selected to evaluate apoptosis and necrosis. Free SM pre-treatment (Figure 4.19c, 4.19d) showed higher cell density compared with cisplatin without pre-treatment demonstrated that free SM might restrain the killing effects of cisplatin. Pre-treatment with SM-loaded BSCS polymeric micelles (Figure 4.19e, 4.19f) showed less morphological change than cisplatin without pre-treatment. A decrease in bright blue fluorescence indicated reducing of chromatin condensation and abnormal nuclei revealed that SM-loaded BSCS polymeric micelles (both concentration; 50 and 100 μg/mL) could reduce cell apoptosis and necrosis induced by cisplatin. Additionally, SM-loaded BSCS polymeric micelles exhibited greater efficacy than free SM in reduction of cell apoptosis and necrosis. Therefore, SM-loaded BSCS polymeric micelles have a potential to prevent cisplatin-induced cell apoptosis and necrosis.

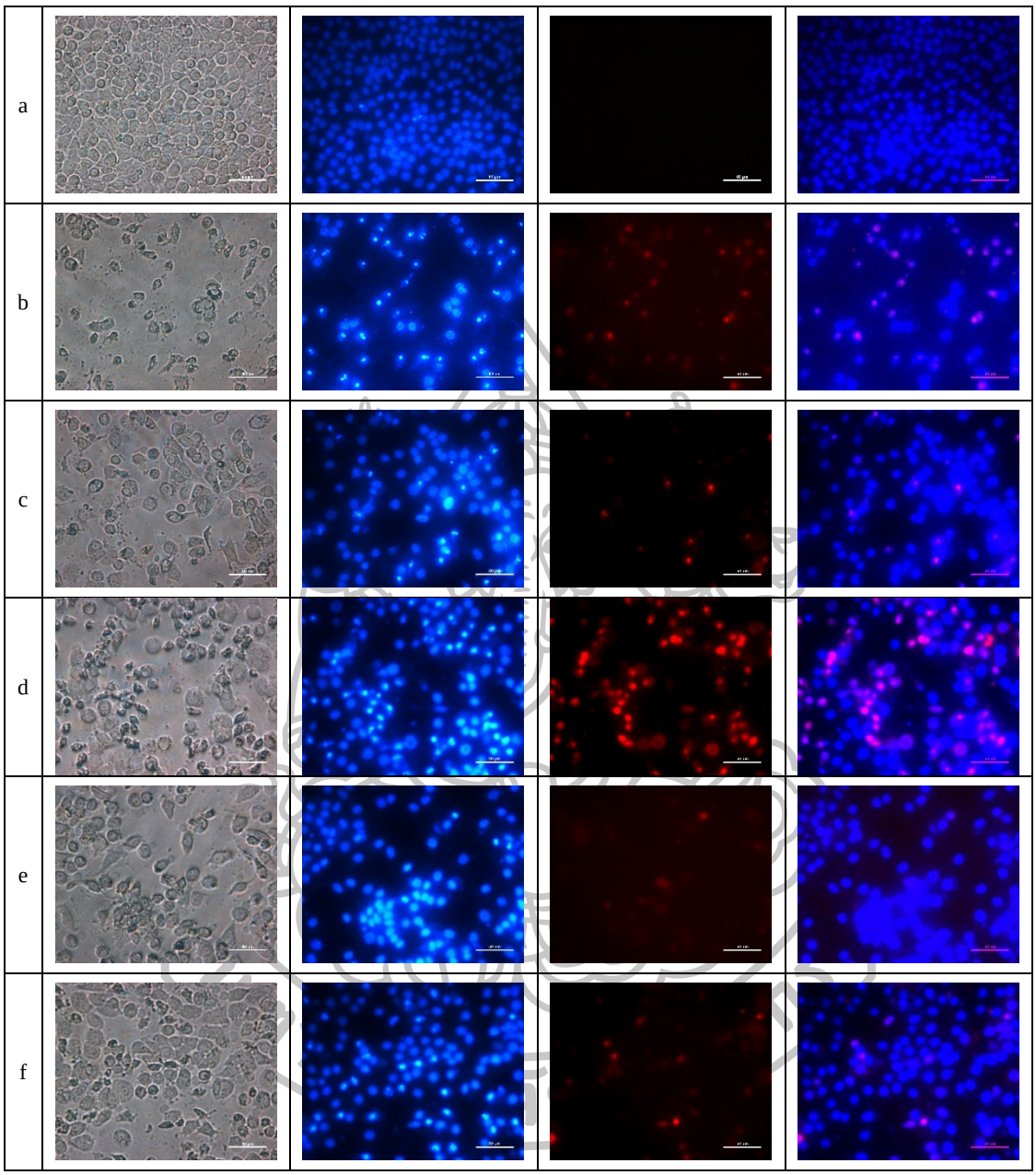


Figure 4.19 Morphology and fluorescence images (40x) of RPTEC/TERT1 cells with different treatment (a) control; (b) cisplatin 50 µM, (c) free SM 50 µg/mL pre-treat and cisplatin 50 µM; (d) free SM 100 µg/mL pre-treat and cisplatin 50 µM; (e) SM-BSCS 50 µg/mL pre-treat and cisplatin 50 µM; (f) SM-BSCS 100 µg/mL pre-treat and cisplatin 50 µM

CHAPTER 5
CONCLUSIONS

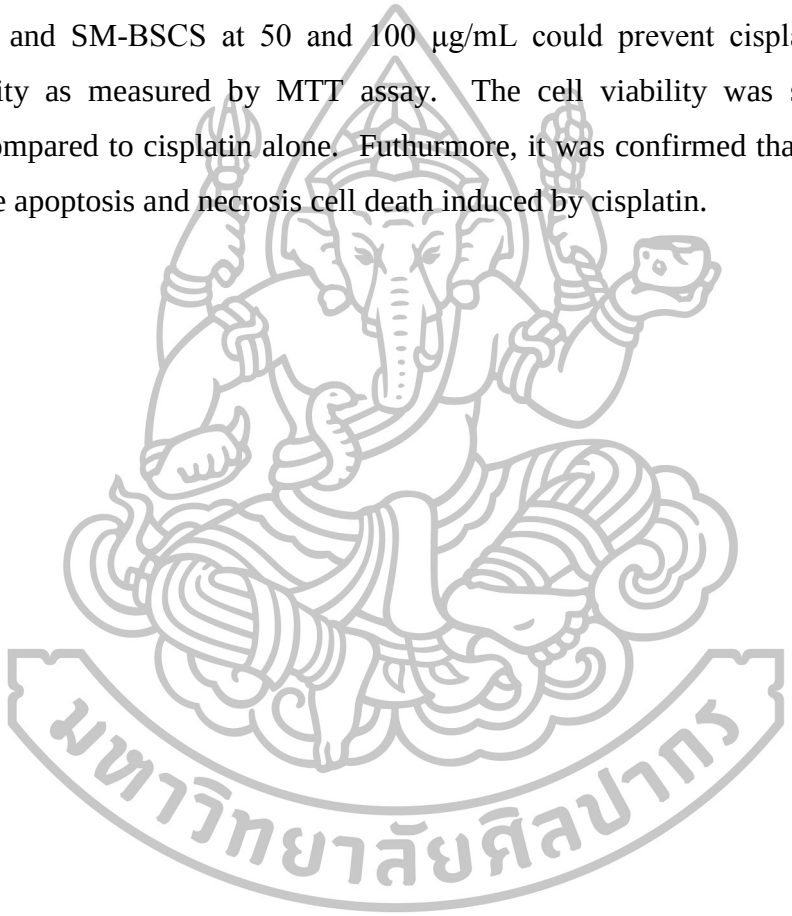
In this present study, SM-loaded polymeric micelles based on amphiphilic chitosan derivatives; (*N*-benzyl-*N*,*O*-succinyl chitosan (BSCS); *N*-octyl-*N*-*O*-succinyl chitosan (OSCS); *N*-naphthyl-*N*,*O*-succinyl chitosan (NSCS) were developed. The polymeric micelles properties, release and permeation behavior, cytotoxicity in HN22 cells and RPTEC/TERT1 cells, killing effects and renoprotective effects were evaluated. The results could be concluded as follow:

SM-loaded polymeric micelles was successfully prepared by evaporation and sonication method as well as cosolvent evaporation method. An increase of initial amount of SM reduced the entrapment efficiency whereas the loading capacity was increased. In the evaporation and sonication method, hydrophobic moieties of polymer played important roles in the entrapment efficiency and loading capacity. The entrapment efficiency of SM-BSCS, SM-NSCS, SM-OSCS was 43-54%, 63-67% and 63-82%, respectively and the loading capacity was 107-257, 134-330, 164-378 μg/mg, respectively. In contrast, the entrapment efficiency and loading capacity of SM-loaded polymeric micelles prepared via cosolvent-evaporation method were not influenced by hydrophobicity of the moieties in the polymer. All polymeric micelles were in spherical shape with highly negative charges. The SM-NSCS showed highest structure stability.

The release profile of SM in GI medium indicated that all polymeric micelles were pH sensitive which released the drug from the formulations if the medium pH was above pK_a of succinyl groups in chitosan backbone. The % cumulative release of SM from SM-loaded polymeric micelles in medium pH 6.8 and 7.4 was significantly different, compared with free SM. In addition, the permeation across porcine intestinal membrane during 1-4 h of SM-loaded polymeric micelles was higher than free drug. This indicated that amphiphilic chitosan derivatives can be used as a potential SM delivery system.

High dose of SM could inhibit the growth of HN22 cells, and both free SM and SM-loaded polymeric micelles did not lower the efficacy of cisplatin in both pre-treatment and co-treatment group. Moreover, high concentration of SM-OSCS could enhance the killing effects of cisplatin due to the highest toxicity of OSCS polymer among others.

The IC_{50} showed low cytotoxicity of SM in RPTEC/TERT1 cells. Pre-treatment of free SM and SM-BSCS at 50 and 100 μg/mL could prevent cisplatin-induced nephrotoxicity as measured by MTT assay. The cell viability was significantly increased compared to cisplatin alone. Futhurmore, it was confirmed that SM-BSCS could reduce apoptosis and necrosis cell death induced by cisplatin.



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APPENDIX

APPENDIX A

Calculations

Calculation of initial amount of silymarin:

Preparation of silymarin-loaded polymeric micelles

Various amount of initial silymarin (20, 40, 60%w/w to polymer) whereas polymer used in all formulations is 5 mg. 20% of 5 mg is 1 mg, 40% of 5 mg is 2 mg, and 60% of 5 mg is 3 mg.

Dialysis method:

Stock solution:

Concentration of silymarin in DMSO: 1 mg/mL

Table Appendix A.2 Preparation of silymarin-polymer solution in dialysis method.

Initial amount of silymarin	Silymarin stock solution (mL)	DMSO (mL)	Total volume (mL)
20% wt polymer	1	1	2

Evaporation and sonication method:

Stock solution:

Concentration of silymarin in DMF: 10 mg/mL

Table Appendix A.2 Preparation silymarin-polymer solution in evaporation and sonication method.

Initial amount of silymarin	Silymarin stock solution (mL)	DMF (mL)	Acetone (mL)	Total volume (mL)
20% wt polymer	0.10	0.35	0.15	0.60
40% wt polymer	0.20	0.25	0.15	0.60
60% wt polymer	0.30	0.15	0.15	0.60

Dropping method:

Stock solution:

Concentration of silymarin in DMSO: 2 mg/mL

Table Appendix A.3 Preparation of silymarin-polymer solution in dropping method.

Initial amount of silymarin	Silymarin stock solution (mL)	Water (mL)	Total volume (mL)
20% wt polymer	0.5	2.5	3

Cosolvent evaporation method:

Stock solution:

Concentration of silymarin in acetone: 10, 20, 30 mg/mL

Table Appendix A.4 Preparation silymarin solution in cosolvent evaporation method.

Initial amount of silymarin	Blank polymeric micelles solution (mL)	Concentration of silymarin in acetone (mg/mL)	silymarin in acetone (mL)	Total volume (mL)
20% wt polymer	3	10	0.1	3.1
40% wt polymer	3	20	0.1	3.1
60% wt polymer	3	30	0.1	3.1

Calculation of entrapment efficiency (%EE):

$$\%EE = \frac{\text{The amount of determined silymarin in micelles}}{\text{Initial amount of silymarin used for the preparation}} \times 100$$

Calculation of loading capacity (LC):

$$LC = \frac{\text{The amount of determined silymarin in micelles}}{\text{Amount of graft copolymer used for the preparation}}$$

Table Appendix A.5 Entrapment efficiency and loading capacity of silymarin (SM) loading in different polymeric micelles prepared by evaporation and sonication method.

Sample	Amount of SM (% to polymer)	Conc. of SM in PMs (ug/ml)	Weight of PMs (g)	Amount of SM in PMs (ug)	Initial amount of SM (ug)	% EE	Average ± SD	LC	Average ± SD
SM-BSCS	20% (1)	190.692	2.7894	531.915	1000	53.19	52.31 ± 0.63	106.38	104.63 ± 1.26
	20% (2)	191.766	2.8352	543.695	1000	54.37		108.74	
	20% (3)	189.390	2.8187	533.834	1000	53.38		106.77	
	40% (1)	377.037	2.7719	1045.109	2000	52.26	52.87 ± 2.99	209.02	211.46 ± 11.98
	40% (2)	348.769	2.8633	998.631	2000	49.93		199.73	
	40% (3)	398.461	2.7930	1112.902	2000	55.65		222.58	
	60% (1)	477.557	2.8133	1343.512	3000	44.78	47.03 ± 3.71	268.70	282.18 ± 22.24
	60% (2)	481.270	2.7824	1339.086	3000	44.64		267.82	
	60% (3)	422.063	2.7728	1170.295	3000	39.01		234.06	
SM-NSCS	20% (1)	238.290	2.7943	665.854	1000	66.59	66.33 ± 0.53	133.17	132.65 ± 1.05
	20% (2)	235.772	2.8579	673.813	1000	67.38		134.76	
	20% (3)	242.699	2.7835	675.554	1000	67.56		135.11	
	40% (1)	449.677	2.8299	1272.542	2000	63.63	65.77 ± 2.33	254.51	263.10 ± 9.34
	40% (2)	482.078	2.8074	1353.385	2000	67.67		270.68	
	40% (3)	453.593	2.8193	1278.814	2000	63.94		255.76	
	60% (1)	581.885	2.7983	1628.290	3000	54.28	60.70 ± 0.76	325.66	364.21 ± 4.59
	60% (2)	588.319	2.8375	1669.356	3000	55.65		333.87	
	60% (3)	586.309	2.8088	1646.826	3000	54.89		329.37	
SM-OSCS	20% (1)	306.516	2.8238	865.540	1000	86.55	81.88 ± 4.13	173.11	163.76 ± 8.26
	20% (2)	276.958	2.8430	787.390	1000	78.74		157.48	
	20% (3)	286.403	2.8408	813.614	1000	81.36		162.72	
	40% (1)	540.275	2.8114	1518.928	2000	75.95	79.87 ± 3.02	303.79	319.49 ± 12.07
	40% (2)	551.231	2.8442	1567.811	2000	78.39		313.56	
	40% (3)	575.369	2.8422	1635.312	2000	81.77		327.06	
	60% (1)	685.281	2.8264	1936.877	3000	64.56	69.70 ± 1.63	387.38	418.21 ± 9.78
	60% (2)	662.224	2.8013	1855.087	3000	61.84		371.02	
	60% (3)	662.016	2.8255	1870.526	3000	62.35		374.11	

Table Appendix A.6 Entrapment efficiency and loading capacity of silymarin(SM) loading in different polymeric micelles prepared by cosolvent evaporation method.

Sample	Amount of SM (% to polymer)	Conc. of SM in PMs (ug/ml)	Weight of PMs (g)	Amount of SM in PMs (ug)	Initial amount of SM (ug)	% EE	Average ± SD	LC	Average ± SD
SM-BSCS	20% (1)	384.588	2.3265	894.742	1000	89.47	90.83 ± 5.39	178.95	181.66 ± 10.78
	20% (2)	348.984	2.7728	967.664	1000	96.77		193.53	
	20% (3)	315.965	2.7296	862.457	1000	86.25		172.49	
	40% (1)	672.363	2.7248	1832.053	2000	91.60	92.62 ± 1.28	366.41	370.48 ± 5.13
	40% (2)	674.956	2.7320	1843.978	2000	92.20		368.80	
	40% (3)	688.485	2.7324	1881.215	2000	94.06		376.24	
	60% (1)	763.336	2.7505	2099.557	3000	69.99	73.46 ± 3.08	419.91	440.79 ± 18.51
	60% (2)	822.147	2.7683	2275.948	3000	75.86		455.19	
	60% (3)	821.404	2.7225	2236.272	3000	74.54		447.25	
SM-NSCS	20% (1)	331.783	2.7114	899.597	1000	89.96	89.18 ± 1.16	179.92	178.36 ± 2.33
	20% (2)	330.839	2.7124	897.366	1000	89.74		179.47	
	20% (3)	322.954	2.7199	878.402	1000	87.84		175.68	
	40% (1)	718.266	2.6700	1917.770	2000	95.89	93.27 ± 3.22	383.55	373.08 ± 12.88
	40% (2)	708.457	2.6606	1884.921	2000	94.25		376.98	
	40% (3)	670.406	2.6752	1793.470	2000	89.67		358.69	
	60% (1)	839.870	2.7106	2276.552	3000	75.89	75.43 ± 2.16	455.31	452.55 ± 12.99
	60% (2)	797.104	2.7500	2192.036	3000	73.07		438.41	
	60% (3)	844.967	2.7453	2319.687	3000	77.32		463.94	
SM-OSCS	20% (1)	319.061	2.7304	871.165	1000	87.12	84.70 ± 4.06	174.23	169.41 ± 8.13
	20% (2)	323.743	2.6868	869.833	1000	86.98		173.97	
	20% (3)	297.249	2.6917	800.106	1000	80.01		160.02	
	40% (1)	685.377	2.6871	1841.677	2000	92.08	91.23 ± 1.19	368.34	364.91 ± 4.74
	40% (2)	670.255	2.6818	1797.490	2000	89.87		359.50	
	40% (3)	686.129	2.6737	1834.502	2000	91.73		366.90	
	60% (1)	798.449	2.7500	2195.734	3000	73.19	74.29 ± 1.11	439.15	445.71 ± 6.64
	60% (2)	793.202	2.8087	2227.868	3000	74.26		445.57	
	60% (3)	816.945	2.7690	2262.119	3000	75.40		452.42	

APPENDIX B

In vitro drug release study and permeation study

Table Appendix B.1 %Drug release in SGF (pH 1.2) 2 h changed to SIF (pH 6.8) to 6 h.

Sample	Time (h)	% Drug release				
		n 1	n 2	n 3	Average	SD
SM-BSCS	0	0.000000	0.000000	0.000000	0.000000	0.000000
	1	4.468245	6.919614	5.785702	5.724520	1.226830
	2	19.491002	14.252509	14.070621	15.938044	3.078296
	3	49.606338	30.006421	31.658632	37.090464	10.870500
	4	37.146879	50.532757	49.760823	45.813486	7.515420
	6	62.545400	59.797101	56.611443	59.651315	2.969664
SM-NSCS	0	0.000000	0.000000	0.000000	0.000000	0.000000
	1	2.578627	5.099847	0.000000	2.559491	2.549977
	2	13.650088	18.222147	14.180607	15.350947	2.500640
	3	47.752126	37.686034	32.977993	39.472051	7.547261
	4	58.332822	41.679551	52.829533	50.947302	8.484689
	6	68.589564	61.340201	50.083595	60.004453	9.325014
SM-OSCS	0	0.000000	0.000000	0.000000	0.000000	0.000000
	1	6.469499	3.579896	6.203612	5.417669	1.597101
	2	24.912131	26.554524	14.158923	21.875192	6.732754
	3	48.990863	50.992429	64.389283	54.790858	8.372508
	4	70.696764	59.112823	38.221068	56.010218	16.458655
	6	74.413347	66.765956	57.470601	66.216635	8.484720
SM	0	0.000000	0.000000	0.000000	0.000000	0.000000
	1	0.000000	0.000000	0.000000	0.000000	0.000000
	2	4.926914	8.071030	3.890557	5.629500	2.176996
	3	14.905723	19.542469	13.551105	15.999766	3.141944
	4	18.003297	32.108334	26.910783	25.674138	7.133371
	6	31.249420	38.193205	33.163330	34.201985	3.586522

Table Appendix B.2 %Drug release in phosphate buffer (pH 7.4) for 24 h.

Sample	Time (h)	% Drug release				
		n 1	n 2	n 3	Average	SD
SM-BSCS	0	0	0	0	0	0
	0.5	24.964372	33.118332	25.983143	28.021949	4.442894
	1	50.126460	70.493901	58.441378	59.687246	10.240718
	2	73.243085	87.492769	76.982073	79.239309	7.388147
	4	80.416544	98.376930	82.563826	87.119100	9.808504
	24	72.245000	86.351000	72.905590	77.167197	7.960262
SM-NSCS	0	0	0	0	0.000000	0.000000
	0.5	12.287919	24.547071	20.433965	19.089652	6.239157
	1	40.171739	54.090786	51.185345	48.482623	7.342581
	2	74.099447	73.115827	65.277279	70.830851	4.834615
	4	82.331575	87.252894	73.539357	81.041275	6.947225
	24	71.846600	69.464000	65.336000	68.882200	3.294062
SM-OSCS	0	0	0	0	0.000000	0.000000
	0.5	24.123470	26.254000	37.678111	29.351860	7.289008
	1	54.548455	60.551270	72.420522	62.506749	9.095088
	2	75.497764	71.960421	88.382584	78.613590	8.643098
	4	80.731323	83.546591	93.661859	85.979924	6.800038
	24	72.867210	76.521290	86.745680	78.711393	7.193775
SM	0	0	0	0	0.000000	0.000000
	0.5	11.368927	7.352660	8.583922	9.101836	2.057614
	1	32.677491	24.288990	28.103119	28.356533	4.199988
	2	52.435642	40.352745	45.807587	46.198658	6.050934
	4	63.681239	52.611884	58.197458	58.163527	5.534755
	24	59.165945	58.598178	52.671932	56.812018	3.596641

Table Appendix B.2 Amount of drug permeate across porcine intestinal membrane in phosphate buffer (pH 7.4) with sodium lauryl sulfate 2% for 4 h.

Sample	Time (h)	Amount of drug permeate across porcine intestinal membrane				
		n 1	n 2	n 3	Average	SD
SM-BSCS	0	0	0	0	0	0
	0.5	0.8838602	0	0	0.2946201	0.5102969
	1	11.9675	27.965088	23.525255	21.152614	8.2584972
	2	35.428415	57.457642	57.641086	50.175715	12.771865
	4	79.225118	94.585045	100.21683	91.342333	10.865055
SM-NSCS	0	0	0	0	0	0
	0.5	1.0123527	0.9378798	0.9843027	0.9781784	0.0376123
	1	14.724216	12.345296	10.304258	12.457923	2.2121304
	2	41.244831	37.798222	31.487053	36.843368	4.9484711
	4	86.526938	77.622565	73.488279	79.212594	6.6631674
SM-OSCS	0	0	0	0	0	0
	0.5	0	0	0	0	0
	1	21.352701	13.820528	30.030867	21.734699	8.1119183
	2	50.411188	34.830933	58.519571	47.920564	12.039115
	4	93.624814	90.770561	93.322709	92.572694	1.5679866
SM	0	0	0	0	0	0
	0.5	1.0424639	1.0518447	11.777726	4.6240117	6.1953004
	1	10.261649	1.0518447	31.167957	14.160483	15.431971
	2	27.884953	26.028556	31.167957	28.360489	2.6024911
	4	61.597707	67.36648	84.936449	71.300212	12.156476

APPENDIX C

Cytotoxicity of polymeric micelles

1. Cytotoxicity of blank polymeric micelles on HN22 cells

1.1 Blank BSCS polymeric micelles

Table Appendix C.1 Cytotoxicity of blank BSCS polymeric micelles.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		300	500	800	1000	2000	3000	4000
1	113.858	99.952	82.125	38.388	32.327	29.594	28.167	30.069
2	98.289	98.170	84.502	46.470	33.278	29.712	32.684	27.811
3	102.924	104.112	94.842	42.905	31.614	27.811	29.237	26.385
4	89.137	88.899	80.937	37.200	32.802	29.356	27.335	27.335
5	95.793	109.936	107.797	46.351	32.446	29.475	30.782	28.286
Average	100.000	100.214	90.040	42.263	32.493	29.189	29.641	27.977
SD	9.209	7.778	11.342	4.344	0.615	0.782	2.134	1.364

1.2 Blank NSCS polymeric micelles

Table Appendix C.2 Cytotoxicity of blank NSCS polymeric micelles.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		300	500	800	1000	2000	3000	4000
1	109.279	61.064	58.904	33.091	27.405	28.315	26.836	27.860
2	101.660	80.623	77.439	33.432	24.448	27.405	28.883	28.428
3	103.593	88.811	88.242	36.161	33.773	28.770	28.997	24.790
4	101.888	83.125	77.325	35.251	27.974	27.405	28.656	28.770
5	83.580	83.239	77.780	31.840	29.224	27.974	30.362	29.679
Average	100.000	79.372	75.938	33.955	28.565	27.974	28.747	27.905
SD	9.682	10.664	10.596	1.735	3.399	0.591	1.260	1.863

1.3 Blank OSCS polymeric micelles

Table Appendix C.3 Cytotoxicity of blank OSCS polymeric micelles.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		300	500	800	1000	2000	3000	4000
1	117.432	71.967	27.562	31.213	30.978	31.567	29.093	30.742
2	105.300	84.452	30.742	31.095	31.802	30.978	32.155	30.742
3	102.120	89.046	29.918	31.095	30.978	29.682	32.155	28.622
4	70.671	77.385	31.802	32.744	29.918	28.386	28.857	29.446
5	104.476	76.678	25.206	26.620	31.331	30.271	30.624	26.148
Average	100.000	79.906	29.046	30.554	31.001	30.177	30.577	29.140
SD	17.440	6.782	2.654	2.308	0.694	1.228	1.593	1.900

2. Cytotoxicity of silymarin-loaded polymeric micelles on Head and Neck cancer cells

2.1. Silymarin-loaded BSCS polymeric micelles

Table Appendix C.4 Cytotoxicity of SM-BSCS against HN22 cells.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		25	50	75	100	200	300	400
1	103.866	94.047	87.450	75.330	77.017	74.102	52.624	45.566
2	98.343	89.291	91.132	84.382	68.733	75.637	53.237	47.254
3	99.877	85.456	89.751	77.938	65.818	70.574	47.867	44.339
4	99.264	86.376	88.984	87.143	70.267	69.500	49.708	40.657
5	98.650	80.853	75.637	75.330	65.818	66.585	45.873	45.413
Average	100.000	87.205	86.591	80.025	69.531	71.280	49.862	44.646
SD	2.240	4.880	6.266	5.434	4.604	3.628	3.120	2.462

2.2. Silymarin-loaded NSCS polymeric micelles

Table Appendix C.5 Cytotoxicity of SM-NSCS against HN22 cells.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		25	50	75	100	200	300	400
1	106.968	79.163	68.204	72.620	77.527	66.405	41.053	42.852
2	99.117	93.883	86.850	68.368	64.279	73.111	42.362	42.035
3	101.734	91.593	77.036	72.130	62.807	64.933	39.908	44.325
4	96.827	95.846	77.854	66.078	60.353	54.138	39.908	43.343
5	95.355	86.359	65.096	62.152	58.554	58.718	40.563	39.091
Average	100.000	89.369	75.008	68.270	64.704	63.461	40.759	42.329
SD	4.582	6.717	8.616	4.361	7.499	7.304	1.017	1.991

2.3. Silymarin-loaded OSCS polymeric micelles

Table Appendix C.6 Cytotoxicity of SM-OSCS against HN22 cells.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		25	50	75	100	200	300	400
1	99.726	85.782	72.958	67.356	59.885	40.588	27.515	29.009
2	120.020	98.855	86.653	58.391	56.275	38.845	28.013	29.507
3	84.412	84.537	74.452	61.255	50.921	42.580	24.651	27.390
4	113.172	88.645	84.786	55.154	50.299	35.234	24.402	27.017
5	82.669	84.163	69.846	56.150	53.536	34.985	24.278	27.141
Average	100.000	88.396	77.739	59.661	54.183	38.446	25.772	28.013
SD	16.716	6.105	7.501	4.899	3.970	3.321	1.832	1.158

3. Cytotoxicity of silymarin on Head and Neck cancer cells

Table Appendix C.7 Cytotoxicity of silymarin.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		25	50	75	100	200	300	400
1	101.040	87.306	67.000	54.934	64.352	49.245	35.609	39.925
2	97.410	89.955	71.316	55.229	58.760	45.321	36.884	33.647
3	99.961	88.974	59.741	56.013	54.640	45.713	37.081	31.195
4	106.631	95.644	75.437	56.504	55.523	41.299	30.901	30.901
5	94.958	71.022	58.662	48.362	55.915	47.087	31.881	35.609
Average	100.000	86.580	66.431	54.208	57.838	45.733	34.471	34.255
SD	4.393	9.245	7.253	3.327	3.955	2.915	2.889	3.707

4. Cytotoxicity of cisplatin on Head and Neck cancer cells

Table Appendix C.8 Cytotoxicity of cisplatin.

n	% Cell viability							
	Control	Concentration (μg/ml)						
		2	4	6	8	10	20	50
1	103.511	92.596	81.408	80.771	57.577	43.933	37.020	16.191
2	100.236	92.323	83.318	68.947	58.668	45.752	38.476	16.554
3	101.510	93.778	82.045	72.767	60.033	41.932	34.928	12.916
4	98.326	89.412	80.226	68.310	57.395	44.297	32.563	15.918
5	96.416	83.318	78.861	67.128	64.490	47.299	39.658	14.281
Average	100.000	90.286	81.172	71.585	59.633	44.643	36.529	15.172
SD	2.753	4.213	1.707	5.553	2.912	2.016	2.833	1.532

APPENDIX D

HPLC chromatogram

Silymarin HPLC chromatogram

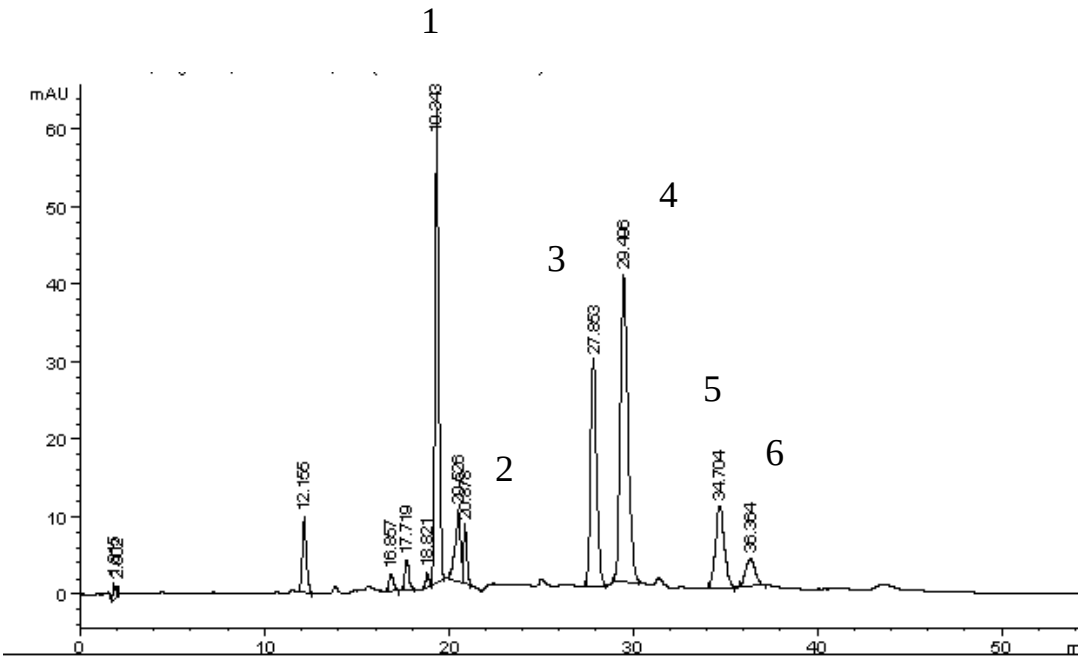

Figure D.1 Silymarin HPLC chromatogram.

Peak number	Components of silymarin
1	Silychristin (marker)
2	Silydianin
3	Silybin A
4	Silybin B
5	Isosilybin A
6	Isosilybin B

Silymarin components:	silybin 60-70%
[7, 10-11]	silychristin 20%
	silydianin 10%
	isosilybin 5%

Standard curve

Table Appendix D.1 Peak area of silymarin components.

Concentration of silymarin (µg/mL)	Peak area			
	Silychristin	Silybin A	Silybin B	Sum
50	132.45667	103.7569	170.30769	406.52126
100	250.99156	197.25182	326.96835	775.21173
150	375.89001	292.10287	485.58783	1153.58071
200	503.61517	390.45987	650.41333	1544.48837
250	624.97589	485.40369	813.60052	1923.9801
300	742.3476	576.9165	965.66125	2284.92535
350	877.9657	681.91895	1147.6698	2707.55445

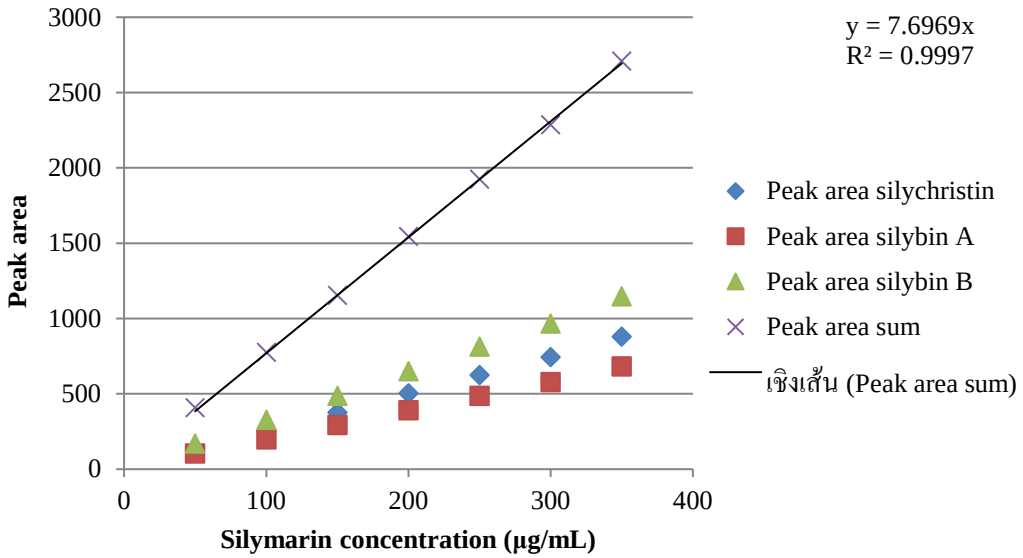

Figure D.2 Silymarin standard curve.

Table Appendix D.2 Peak area of silychristin.

Concentration of silymarin (µg/mL)	Peak area of Silychristin
5	11.78338
10	21.35356
20	46.08792
40	91.66122
80	174.52429

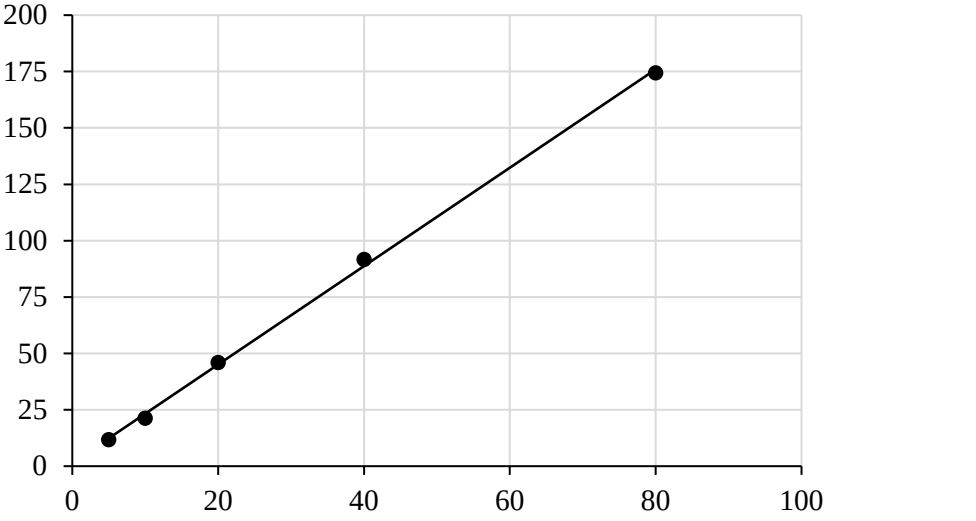

Figure D.3 Silychristin standard curve.

BIOGRAPHY

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Poster Presentation

1. **Wajee Tipparos**, Warayuth Sajomsang, Tanasait Ngawhirunpat, Theerasak Rojanarata, Prasert Akkaramongkolporn and Praneet Opanasopit. "Development of silymarin-loaded polymeric micelles based on amphiphilic chitosan derivatives", 32nd International Annual Meeting in Pharmaceutical Sciences. 10-11 March 2016, Chulalongkorn University, Bangkok, Thailand.
2. **Wajee Tipparos**, Warayuth Sajomsang, Tanasait Ngawhirunpat, Theerasak Rojanarata, Prasert Akkaramongkolporn and Praneet Opanasopit. "Preparation and characterization of silymarin-loaded amphiphilic chitosans micelles", The 4th Current Drug Development International Conference (CDD 2016). 1-3 May 2016, Phuket, Thailand.
3. **Wajee Tipparos**, Warayuth Sajomsang, Tanasait Ngawhirunpat, Theerasak Rojanarata, Prasert Akkaramongkolporn and Praneet Opanasopit. "*In vitro* release and intestinal permeation of silymarin-loaded polymeric micelles", 26th FAPA Congress 2016. 9-13 November 2016, Bangkok, Thailand.