การพัฒนาโพรลิโพโซมเพื่อการนำส่งซับสเตรตของพี-ไกลโคโปรตีน โดยการรับประทาน

นางสาวเกศินี เนตรสมบูรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

DEVELOPMENT OF PROLIPOSOMES FOR ORAL DELIVERY OF P-GLYCOPROTEIN SUBSTRATES

Miss Kesinee Netsomboon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutics
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2011

Thesis Title	DEVELOPMENT OF PROLIPOSOMES FOR ORAL DELIVERY OF P-GLYCOPROTEIN SUBSTRATES
By	Miss Kesinee Netsomboon
Field of Study	Pharmaceutics
Thesis Advisor	Assistant Professor Nontima Vardhanabhuti, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Associate Professor Uthai Suvanakoot, Ph.D.)

…….……. Thesis Advisor

(Assistant Professor Nontima Vardhanabhuti, Ph.D.)

…….. Examiner

(Associate Professor Waraporn Suwakul, Ph.D.)

…….. External Examiner

(Associate Professor Nusara Piyapolrungroj, Ph.D.)

เกศินี เนตรสมบูรณ์ : การพัฒนาโพรลิโพโซมเพื่อการนำส่งซับสเตรตของพี-ไกลโคโปรตีนโดยการรับประทาน. (DEVELOPMENT OF PROLIPOSOMES FOR ORAL DELIVERY OF P-GLYCOPROTEIN SUBSTRATES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.นนทิมา วรรธนะภูติ, 103 หน้า

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ในการนำระบบนำส่งยารูปแบบโพรลิโพโซมมาใช้เพื่อเพิ่มการนำส่งซับสเตรตของพี-ไกลโคโปรตีนโดยการรับประทาน ในการศึกษานี้ใช้แคลซีนเอเอ็มและอะซัยโคลเวียเป็นตัวแทนของซับสเตรตที่ชอบไขมันและชอบน้ำตามลำดับ แบบจำลองของเยื่อบุลำไส้เล็กที่ใช้ในการศึกษานี้คือเซลล์คาโค-2 วิธีที่ใช้เตรียมโพรลิโพโซมคือการทำให้เกิดฟิล์มของฟอสโฟลิพิดบนอนุภาคของตัวพา ซึ่งในการศึกษานี้ใช้ซอร์บิทอล นำโพรลิโพโซมที่มีคุณสมบัติทางกายภาพ (ได้แก่ ลักษณะที่ปรากฏ, สัณฐานวิทยาของพื้นผิว และคุณสมบัติการไหล) ที่เหมาะสมและสามารถเกิดเป็นลิโพโซมเมื่อสัมผัสกับเฟสน้ำไปใช้ในการทดลองเพื่อประเมินการเพิ่มการนำส่งซับสเตรตของพี-ไกลโคโปรตีน ความสามารถในการเพิ่มการนำส่งซับสเตรตของพีไกลโคโปรตีนประเมินโดยการเปรียบเทียบปริมาณสะสมของซับสเตรตของพี-ไกลโคโปรตีนในเซลล์คาโค-2 ปริมาณการสะสมของแคลซีนเอเอ็มวิเคราะห์ได้โดยวิธีสเปคโตรฟลูออโรเมตริก ที่ความยาวคลื่น 485/535 นาโนเมตร การวิเคราะห์หาปริมาณของอะซัยโคลเวียทำโดยใช้โครมาโทกราฟีของเหลวสมรรถนะสูงที่ต่อกับยูวีดีเทคเตอร์ที่ความยาวคลื่น 254 นาโนเมตร ผลการศึกษาพบว่าสามารถบรรจุฟอสฟาทิดิลโคลีนจากถั่วเหลืองลงในตัวพาปริมาณหนึ่งกรัมได้เท่ากับ 0.20 มิลลิโมล อัตราส่วนระหว่างฟอสฟาทิดิลโคลีนจากถั่วเหลืองต่อโคเลสเทอรอลที่ทำให้โพรลิโพโซมมีลักษณะทางกายภาพตามที่กำหนดได้แก่ อัตราส่วน 1:0 และ 1:0.25 โดยโมล การศึกษาผลการใช้โพรลิโพโซมเพื่อเพิ่มการนำส่งซับสเตรตของพีไกลโคโปรตีนในเซลล์คาโค-2 พบว่าโพรลิโพโซมที่บรรจุแคลซีนเอเอ็มทั้งสูตรที่มีและไม่มีโคเลสเทอรอลสามารถเพิ่มการนำส่งแคลซีนเอเอ็มเข้าไปในเซลล์คาโค-2 ได้มากกว่าสองเท่าเมื่อเปรียบเทียบกับการนำส่งในรูปแบบสารละลาย ผลเพิ่มการนำส่งโดยโพรลิโพโซมที่ไม่มีโคเลสเทอรอลใกล้เคียงกับการนำส่งในรูปแบบของลิโพโซมที่ลดขนาดด้วยการกดผ่านเมมเบรนที่ทำจากโพลีคาร์บอเนตซึ่งมีช่องเปิดขนาด 100 นาโนเมตร โพรลิโพโซมที่บรรจุแคลซีนเอเอ็มที่มีโคเลสเทอรอลเป็นส่วนประกอบสามารถเพิ่มการนำส่งแคลซีนเอเอ็มได้มากกว่าสูตรที่ไม่มีโคเลสเทอรอล ในทางตรงกันข้ามไม่พบผลดีจากการใช้โพรลิโพโซมเพื่อเพิ่มการสะสมของอะซัยโคลเวียในเซลล์คาโค-2 ที่คาดว่าจะเกิดจากกลไกการหลีกเลี่ยงการทำงานของพี-ไกลโคโปรตีนและ/หรือจากกระบวนการเอนโดไซโตซิส โพรลิโพโซมที่บรรจุอะซัยโคลเวียไม่สามารถเพิ่มการสะสมของอะซัยโคลเวียในเซลล์คาโค-2 ได้ ส่วนหนึ่งอาจเป็นผลจากอะซัยโคลเวียถูกกักเก็บในลิโพโซมได้น้อย (น้อยกว่าร้อยละ 5 ของปริมาณอะซัยโคลเวียทั้งหมด) ข้อมูลจากการศึกษานี้บ่งชี้ว่าการใช้ระบบนำส่งรูปแบบโพรลิโพโซมเหมาะสำหรับสารที่ชอบไขมัน สำหรับสารที่ชอบน้ำอาจจำเป็นต้องเพิ่มปริมาณการกักเก็บสารในลิโพโซม และการนำโพรลิโพโซมไปใช้นำส่งสารที่เป็นซับสเตรตของพี-ไกลโคโปรตีนที่ชอบน้ำจะได้ผลหรือไม่ อาจขึ้นกับกลไกในการเข้าเซลล์ของสารแต่ละตัวด้วย ดังนั้นจึงควรมีการศึกษาเพิ่มเติมในเรื่องดังกล่าวในการพัฒนาโพรลิโพโซมต่อไป

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2554.....

5176612133: MAJOR PHARMACEUTICS
KEYWORDS: P-glycoprotein/Proliposomes/Caco-2/Acyclovir/Calcein AM

KESINEE NETSOMBOON: DEVELOPMENT OF PROLIPOSOMES FOR ORAL DELIVERY OF P-GLYCOPROTEIN SUBSTRATES. ADVISOR: ASST. PROF. NONTIMA VARDHANABHUTI, Ph.D., 103 pp.

The purpose of this study was to examine the feasibility of using proliposome delivery systems to improve the delivery of P-glycoprotein (P-gp) substrates via the oral route. Calcein AM and acyclovir were used as the hydrophobic and the hydrophilic models for P-gp substrates, respectively. Caco-2 cells were used as the intestinal epithelium model. Proliposomes were prepared by film deposition on carriers. Sorbitol particles were used as the carrier. The optimum proliposome compositions were determined from the physical properties (physical appearance, surface morphology, and flow properties) of the proliposome particles as well as the formation of liposomes upon hydration of the proliposomes with the aqueous phase. The ability of proliposomes to enhance the delivery of P-gp substrates was evaluated from the accumulation of the model substrates in Caco-2 cells. Intracellular accumulation of calcein AM was determined by the spectrofluorometric method at the excitation and emission wavelengths of 485 and 535 nm, respectively. Acyclovir was determined by the high performance liquid chromatographic method with a UV detector at 254 nm. The maximum amount of soybean phosphatidylcholine (SPC) that could be loaded onto the sorbitol particles was 0.20 mmol/g of sorbitol. The molar ratios of SPC:CHO that gave satisfactory proliposome preparations were 1:0 and 1:0.25. Calcein AM-loaded proliposomes, both with and without CHO, could significantly enhance the accumulation of calcein AM in Caco-2 cells by more than two fold when compared with the solution. The degree of enhancement of proliposomes without CHO was comparable to that of the liposomes extruded through 100-nm polycarbonate membranes. Inclusion of CHO in proliposomes further enhanced the uptake of calcein AM into Caco-2 cells. On the contrary, the expected benefits of proliposomes on acyclovir accumulation either via the bypass of P-gp function or by the increase in the uptake via endocytosis were not seen. Proliposome preparations could not enhance the intracellular accumulation of acyclovir in Caco-2 cells. Low entrapment efficiency (less than 5% of total acyclovir) might be partly responsible. These results suggested that the use of proliposome delivery systems to enhance the delivery of P-gp substrates might be feasible for hydrophobic molecules. In order to be successful with hydrophilic P-gp substrates, a strategy to increase entrapment efficiency might be necessary. The success of proliposome formulations in enhancing the delivery of hydrophilic P-gp substrates might also depend on the available mechanism(s) of uptake of each individual substrate. These issues should be addressed for further study on proliposomes.

Department: Pharmaceutics and Industrial Pharmacy Student's Signature .....
Field of Study: Pharmaceutics Advisor's Signature .....
Academic Year: 2011

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my advisor, Assistant Professor Nontima Vardhanabhuti, Ph.D., for her scientific guidance, kindness, encouragement, and understanding throughout this study.

I would also like to thank the thesis committee for their valuable suggestions and helpful discussion.

I sincerely thank the scholarship from Chula-Chiba University Pharmaceutical Student Exchange Program. Sincere gratitude is also directed to Professors and staff members of the Department of Pharmaceutical Technology, Graduate School of Pharmaceutical Sciences, Chiba University, Japan, for their support for the works done on the scanning electron microscope.

I am deeply thankful to Associate Professor Nusara Piyapolrungroj, Ph.D., for her guidance in cell culture techniques. I would also like to thank Associate Professor Titinan Auamnoy, Ph.D., for his guidance in statistical analysis. I am also thankful to Miss Ing-orn Prasanchaimontri for her helpful suggestions on cell culture experiments.

I also sincerely thank Assistant Professor Suree Jianmongkol, Ph.D., and the T.O. Chemicals (1979) Co., Ltd., for the gifts of cyclosporine A and acyclovir, respectively. Thanks are also due to Professor Garnpimol C. Ritthidej, Ph.D., for the use her research facilities.

I sincerely thank the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Higher Education Research Promotion and National Research University Project of Thailand (AS638A) for their partial financial supports.

I would also like to thank the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their support. Thanks are also directed to the Department of Biochemistry and Microbiology for the use of equipment.

I also thank the Department of Pharmaceutics and Industrial Pharmacy for the support of scientific instruments and utilities. Its staff members are also thanked for their kind support throughout my graduate study.

Sincere thanks are also given to all my friends in the Department of Pharmaceutics and Industrial Pharmacy, the Department of Pharmacology and Physiology, and to other persons whose names have not been mentioned for their friendship and valuable help. In addition, I would like to thank all the faculty members in both departments for their support and encouragement.

Above all, I would like to express my deepest gratitude to my family for the unconditional encouragement, care, love, and support given to me throughout the years.

CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	=	analysis of variance
BCRP	=	breast cancer resistant protein
BCS	=	biopharmaceutics classification system
ca	=	circa
Caco-2	=	human colon adenocarcinoma
CHO	=	cholesterol
cm^2	=	square centimeter
cm^3	=	cubic centimeter
CYP	=	cytochromes P450
Da	=	Dalton
DMEM	=	Dulbecco's Modified Eagle's Medium
DMSO	=	dimetyl sulphoxide
EDTA	=	ethylenediaminetetraacetic acid
EE	=	entrapment efficiency
ENT	=	Na^+-independent equilibrative nucleoside transporter
g	=	gram
GI	=	gastrointestinal
HEPES	=	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HPLC	=	high performance liquid chromatography
kV	=	kilovolt
m^2	=	square meter
MDR1	=	multidrug resistance protein 1
MEM	=	Eagle's Minimal Essential Medium
mg	=	milligram
ml	=	milliliter
mM	=	millimolar
mmol	=	millimole

MRP	=	multidrug resistance-associated protein
mSIF	=	modified simulated intestinal fluid
N	=	normal
nm	=	nanometer
nmol	=	nanomole
nM	=	nanomolar
OCT1	=	organic cation transporter1
PBS	=	phosphate buffered saline
P-gp	=	P-glycoprotein
rpm	=	round per minute
SD	=	standard deviation
SEM	=	standard error of means
SIF	=	simulated intestinal fluid
SPC	=	soybean phosphatidylcholine
TS	=	test solution
µg	=	microgram
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

Oral route is the most preferred route of drug administration. Orally administered drugs have to pass through both physical and biochemical barriers in the gastrointestinal (GI) tract before entering the systemic circulation, where they are further delivered to and exert their therapeutic effects at their specific targets. In the absorption process, drugs should be dissolved in the GI fluid and subsequently permeate through the intestinal epithelium. The efficiency of the absorption process also depends partly on how well the drug molecules can overcome the two types of barrier on the epithelium membrane. Those two types of barrier are physical and biochemical barriers.

The physical barrier is one of the factors that affect the efficiency of the absorption process. There are two main absorption pathways through the physical barrier of the GI tract. These are the paracellular and the transcellular pathways (Hayashi, Tomita, and Awazu, 1997; Chan, Lowes, and Hirst, 2004). For the paracellular pathway, drug molecules are absorbed by passing through the tight junctions. Drugs that can be absorbed via this pathway are mostly small, water-soluble molecules such as atenolol, ranitidine, cimetidine, and hydrochlorothiazide (Collett et al., 1996). However, the cross-sectional area of the tight junction is only about 0.01% of the histological surface of the villi (Pappenheimer, 1987). Because of the limited absorption area and the special molecular property requirements, this pathway is not the major absorption pathway for most drugs.

For the transcellular pathway, the drug molecules are absorbed through the enterocytes. There are two types of mechanism available for drug transport transcellularly. These are the passive diffusion and the carrier-mediated transport (Chan et al., 2004). The passive diffusion is the transport mechanism of most drugs administered via the oral route. For drug molecules to be absorbed by passive diffusion, some specific physicochemical properties such as lipophilicity, proper surface charges, appropriate molecular volume/weight, and conformational flexibility are required (Ungell and Abrahamsson, 2001). On the other hand, drug molecules

with structures similar to nutrients can be taken up by the facilitated transport or by the active transport, both of which are classified as carrier-mediated transport systems. Nevertheless, some drugs with the physicochemical properties that meet those requirements still have low oral bioavailability. The GI absorption of these drugs is hindered mostly by the biochemical barrier in the GI tract.

The biochemical barrier in the GI tract comprises the efflux transporters and the metabolizing enzymes. The efflux transporters are found on both the apical and the basolateral membranes of the intestinal epithelium. The function of the efflux transporters is to prevent substrate accumulation in the cells by limiting absorption capacity and increasing their effluxes back into the intestinal lumen (Schinkel and Jonker, 2003; Takano, Yumoto, and Murakami, 2006). Both Phase I and Phase II drug metabolizing enzymes are found in enterocytes (Washington, Washington, and Wilson, 2001; Sambuy et al., 2005). These include the various cytochrome P450 isoforms, UDP-glucuronosyltransferases, sulfotransferases, and glutathione-S-transferases. Though the impact of these metabolizing enzymes on oral drug bioavailability is more prevalent in the liver, the synergistic role of the metabolizing enzymes and the efflux transporters is well evident in enterocytes (Washington et al., 2001). Thus, much effort has been directed to moderate the function of the efflux transporters to increase oral bioavailability of drugs (Jain et al., 2005; Föger, Schmitz, and Bernkop-Schnürch, 2006; Bansal et al., 2009a).

Efflux transporters are expressed in several areas of the human body including small intestine, liver, kidney, and brain. The efflux transporters found in the GI tract include both the multidrug resistance (MDR) and the multidrug resistance-associated protein (MRP) types (Chan et al., 2004). The most widely studied efflux transporter in the GI tract is the P-glycoprotein (P-gp) (Varma, Perumal, and Panchagnula, 2006; Bansal et al., 2009a).

P-gp or multidrug resistance protein 1 (MDR1) has been reported to affect drug bioavailability in many groups of drugs. The expression of P-gp is also found in normal tissues. High expression levels of P-gp have been found in adrenal gland, kidney, colon, liver, lung, prostate, skin, spleen, heart, skeletal muscle, stomach, ovary, breast, brain, choroid plexus, placenta, and also in the small intestine (Chan et al., 2004). Several classes of drugs, e.g. analgesics, anticancer drugs, cardiac drugs,

HIV protease inhibitors, H_2-receptor antagonists, anti-gout agents, antidiarrheal agents, antiemetics, immunosuppressive agents, antibiotics, and steroid hormones are known substrates of the efflux transporter (Schinkel and Jonker, 2003; Chan et al., 2004). These drugs do not have related molecular structures, but most of them have some similar physicochemical properties such as being cationic, lipophilic molecules. Similar to other efflux transporters, P-gp prevents the accumulation of drugs in enterocytes, resulting in drugs remaining in the intestinal lumen. Besides low bioavailability, this can lead to GI irritation as well as anticancer/antibiotic drug resistance (Bansal et al., 2009a).

There are several strategies to improve the intestinal absorption of these P-gp substrate drugs. Development of the novel structures such as conjugation with dendrimer (D'Emanuele et al., 2004) and prodrug modification (Jain et al., 2005) has been implemented. However, toxicity from the incomplete conjugation and limited window of absorption for those prodrugs are still the major concerns (Walter, Kissel, and Amidon, 1996; D'Emanuele et al., 2004). Alternatively, P-gp function modulators such as cyclosporine A, ketoconazole, LY335979, nelfinavir, quinidine, ritonavir, saquinavir, tacrolimus, valspoda (PSC833), verapamil, elacridar (GF120918, GG918), and reserpine have been used (U.S. FDA, 2011). The co-administration of P-gp inhibitors and substrates can increase the accumulation of the substrates. However, this practice often requires high doses of the inhibitors. This can lead to undesired intrinsic pharmacological effects of the inhibitors. The undesired effects are evident with cyclosporine A (Ozben, 2006) and verapamil (Varma et al., 2003). Another strategy to bypass the activity of efflux transporters is the use of drug delivery systems such as liposomes (Mamot et al., 2003; Ing-orn Prasanchaimontri, 2009), micelles (Dabholkar et al., 2006), and nanoparticles (Nassar et al., 2009). Liposomes are widely used for delivering both hydrophilic and hydrophobic drugs. Several studies have shown that liposomal drug delivery systems can bypass the P-gp effect (Lo, Liu, and Cherng, 2001; Mamot et al., 2003; Kobayashi et al., 2007).

However, oral administration of liposomes can result in erratic absorption profiles due to their susceptibility to the gastric acid and the enzymes in the GI tract (Ariën et al., 1993). Moreover, liposomes are both physical and chemical unstable due to the lability of the phospholipid components. Proliposome delivery systems have

been developed to overcome these problems (Payne et al., 1986; Betageri, 2005; Deshmukh, Ravis, and Betageri, 2008).

Proliposomes are defined as dry, free-flowing particles that can form multilamellar liposomes upon contact with the water phase (Payne et al., 1986). Proliposomes have been used to improve solubility (Yan-yu et al., 2006; Hiremath, Soppimath, and Betageri, 2009) and permeability (Deshmukh et al., 2008; Hiremath et al., 2009) of active ingredients. The use of proliposomes to deliver salmon calcitonin into Caco-2 cells can increase the apparent permeability of the polypeptide (Song, Chung, and Shim, 2002). Proliposomes containing bile salts can also increase absorption of salmon calcitonin in Caco-2 cells and in rats (Song, Chung, and Shim, 2005). Moreover, an in vivo study of proliposomes showed that the proliposome delivery system could increase the absorption of silymarin in beagle dogs (Yan-yu et al., 2006). Xu and co-workers (2009) have successfully improved the oral delivery of vinpocetine in New Zealand rabbits. However, none of these studies were done specifically on P-gp substrates. Thus, the aim of the present study was to evaluate whether it would be feasible to use proliposome delivery systems to bypass the function of P-gp. In this study, calcein AM and acyclovir have been used as models for lipophilic and hydrophilic substrates, respectively.

To evaluate the advantages of proliposomes in the delivery of lipophilic P-gp substrates, calcein AM was used as the model compound. Calcein AM is a non-fluorescent lipophilic molecule. It is also a known P-gp substrate that has been used extensively in the field of P-gp research (Pendse, Briscoe, and Frank, 2003; Bubik et al., 2006; Feng et al., 2008). Following the uptake process into the cells, calcein AM interacts with cellular esterases and releases the fluorescent marker calcein, which is easily detected by the spectrofluorometric method. Calcein AM is of a special advantage since it allows easy discrimination between the intact calcein AM outside the cells and the hydrolytic product calcein within the cells.

Acyclovir is an HIV protease inhibitor with a limited oral bioavailability of 15-30% (Lacy et al., 2005). Acyclovir is classified by the biopharmaceutics classification system (BCS) as a class III drug with high solubility and low permeability (Lindenberg, Kopp, and Dressman, 2004). Acyclovir encompasses absorption problems from both the physical and the biochemical barriers. Since

acyclovir is a small hydrophilic molecule, its predominant absorption pathway is the paracellular route (De Vrueh, Smith, and Lee, 1998). However, acyclovir is also absorbed by the saturable carrier-mediated transport. Transporters that are responsible for absorption of acyclovir include the organic cation transporter1 (OCT1) (Takeda et al., 2002) and the Na^+-independent equilibrative nucleoside transporter (ENT) (Franco et al., 2008). Drug absorbed via the carrier-mediated transport are known to have limited windows of absorption (Alam, Al-Jenoobi, and Al-Mohizea, 2012). In addition, acyclovir has been classified as a P-gp substrate, which is believed to be the cause of its low permeability (De Vrueh et al., 1998; Salama, Scott, and Eddington, 2004; Palmberger, Hombach, and Bernkop-Schnürch, 2008). Since liposomes are also known to increase the absorption of hydrophilic compounds (Sheue Nee Ling et al., 2006), proliposomes were expected to be beneficial for the delivery of hydrophilic P-gp substrates such as acyclovir. To investigate the prevailing effect of proliposomes over both the physical and the biochemical barriers of hydrophilic P-gp substrates, acyclovir was selected as the model in this study.

The cell line used in this study was the human epithelial colorectal adenocarcinoma cells, Caco-2. After cell differentiation, polarized epithelial cell monolayers are formed from the cell line. This provides the characteristics similar to the small intestinal lining. Caco-2 cells have been long accepted as an intestinal absorption model (Taubert et al., 2006; Mukaizawa et al., 2009; Wahlang, Pawar, and Bansal, 2011). Caco-2 cells express some transporters that are similar to those found in the intestine, including OCT1 and ENT (Hayeshi et al., 2008). Similar to other tumor cell lines, the Caco-2 cell line also expresses P-gp on its apical surface (Faassen et al., 2003; Engdal and Nilsen, 2008). Because of these properties, Caco-2 cells were chosen for the *in vitro* absorption model for this study.

In this study, proliposomes were prepared by film deposition on carriers adapted from the method used by Song et al. (2002). The formulation composition was systematically varied until the acceptable formulations were obtained. The uptake of the model P-gp substrates into Caco-2 cells from proliposomes was evaluated for the feasibility of using the delivery systems to increase the GI absorption of these compounds.

The specific aims of this study were to evaluate:

1. The effect of proliposome composition on the physical properties of proliposomes and the formation of liposomes from proliposomes
2. The delivery of the model P-gp substrates calcein AM and acyclovir into Caco-2 cells using proliposomes

CHAPTER II

LITERATURE REVIEW

1. Absorption pathways across the intestinal epithelium

Transport of drug molecules across intestinal barriers can be classified into several pathways as depicted in Figure 1. These pathways include passive transcellular and paracellular routes, carrier-mediated transport, carrier-mediated efflux, and vesicular transport.

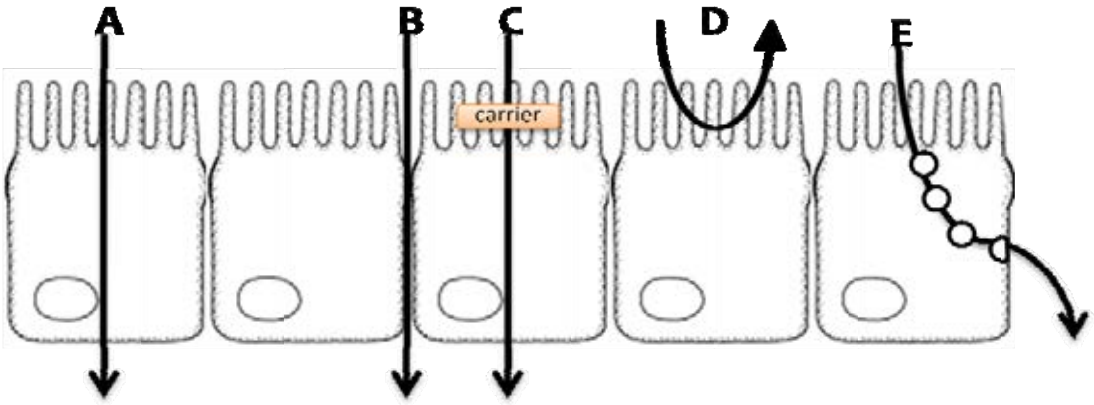


Figure 1: Routes and mechanisms of drug transport across the intestinal epithelium: (A) passive transcellular route, (B) passive paracellular route, (C) carrier-mediated transport, (D) carrier-mediated efflux, and (E) vesicular transport (modified from Jung et al., 2000b; Chan, et al., 2004)

1.1. Passive diffusion (Shargel,Wu-Pong, and Yu, 2005)

Passive diffusion is generally a major absorption process for low molecular weight drugs. The drug molecules are transported along the concentration gradient without consuming energy. When the equilibrium is reached, the concentrations of drug on both sides are equal, resulting in no net transfer of drug. However, in the intestine, the concentration of drug in plasma is much lower than that in the lumen. This high concentration gradient results in forward moving of drug molecules from the lumen across the intestinal epithelium, thus the absorption of the drug. Passive diffusion can be further classified into transcellular and paracellular pathways.

1.1.1. Passive transcellular pathway (Taylor, 1996; Hayashi et al., 1997; Ungell and Abrahamsson, 2001; Shargel et al., 2005)

The transcellular pathway is generally a principal transport route for small molecule drugs with some degree of lipophilicity. Drug molecules are transported across the apical epithelium into the cytoplasm, move to the basolateral membrane and pass through to the other side of the membrane (Figure 1A). Although the passive transcellular pathway is a non-specific process, several physicochemical drug factors influence the absorption, including water and lipid solubility, partition coefficient, degree of hydrogen bonding, and molecular size. In order to predict oral absorption, Lipinski et al. (1997) have suggested the “rule-of-five”. The drug molecules having suitable physicochemical properties for passive transcellular diffusion are those with molecular weights of less than 500 daltons, the number of hydrogen bond donors (O–H or N–H group) of less than 5, number of hydrogen bond acceptors (O or N) of less than 10, and the calculated log *P* of less than 5.

1.1.2. Passive paracellular pathway (Pappenheimer,1987; Hayashi et al., 1997; Daugherty and Mrsny, 1999; Karlsson et al., 1999; Shargel et al., 2005)

Upon absorption by the paracellular pathway (Figure 1B), drug molecules are absorbed through the lateral intercellular space between adjoining epithelial cells. The paracellular pathway is suitable for water soluble, ionized compounds. Substances that can be absorbed via this pathway include creatinine and erythritol. However, the functional cross-sectional area of the tight junction is only about 0.01% of the surface area of the villi. Because of the small area of absorption and the selectivity of charge and molecular size, the paracellular pathway plays a minor role in the absorption process.

1.2. Carrier-mediated transport (Washington, et al., 2001; Shargel et al., 2005; Sharom, 2008)

The carrier-mediated transport is the process for transporting nutrients and other molecules with the structures similar to the nutrients and ions. Transport via this pathway requires carrier proteins that bind specifically with the molecules. The carrier-mediated transport is further classified as active and facilitated transport.

1.2.1. Active transport

The active transport is the transport process against the concentration gradient. This process requires energy for transporting the substances from the lower concentration area to the higher concentration area. If the external concentration of the molecule being transported is extremely high, the carrier will be fully utilized and will become rate limiting. Under this condition, increasing the external concentration of the transported molecule will have no effect on the transport rate.

Active transport can occur in two directions, from apical to basolateral and from basolateral to apical. The transport in the apical to basolateral direction is used to transport the nutrients or substances with the chemical structure similar to the nutrients such as fluorouracil. The transport in the other direction is efflux transport. The efflux transport is the process to prevent the tissues from toxic xenobiotics. Although efflux transporters are mostly expressed in tumor cells, they can be found in normal tissues especially in the sensitive organs such as capillaries in the brain, pregnant endometrium, placenta, and the organs which absorb or eliminate substances such as the intestinal epithelium, pancreatic and bile ductules, and kidney proximal tubules.

1.2.2. Facilitated transport

The facilitated transport also requires carriers, similar to the active transport. However, this process transports the substances along the concentration gradient, and thus no biochemical energy consuming. Since carriers are needed, this transport process is also a saturable process. The substance absorbed via this pathway is small carbohydrate molecules such as glucose.

1.3. Vesicular transport (Shargel et al., 2005)

The vesicular transport is the process of cell engulfing particles or fluid. Vesicular transport can be classified into pinocytosis, phagocytosis, endocytosis, and exocytosis. Pinocytosis is the engulfment of small solutes or fluid, while phagocytosis is the engulfment of larger particles or macromolecules. Phagocytosis occurs in some special cells such as macrophages. On the contrary, endocytosis and exocytosis are the processes of specific macromolecule transport into and out of the cell, which take place in all types of cell in the body.

2. Efflux transporters (Chan et al., 2004; Takano et al, 2006)

Efflux transporters are proteins expressed on the cell membranes. These transporters can be classified into MDR (multidrug resistant)- and MRP (multidrug resistance-associated protein)-type transporters. The function of efflux transporters is to limit the accumulation of substrates in the cells by increasing their efflux out of the cells. This function results in low bioavailability of the substrates. Not only in the tumor cells, efflux transporters are also found in the normal tissues, especially the tissue with absorption (intestine) or elimination (liver and kidney) function. Among the many types of efflux transporter, P-glycoprotein (P-gp or MDR1) is one of the most extensively studied, ATP-dependent efflux transporters.

3. P-glycoprotein efflux transporter (Chan et al., 2004; Takano et al., 2006; Sharom, 2008)

P-gp is a 170 kDa single polypeptide containing 1280 amino acids, with 2 homologous parts of approximately equal length, 2 ATP-binding domains, and 12 transmembrane regions. As shown in Figure 2, the structure of P-gp consists of two blocks. Each block contains six trans-membrane regions and an ATP-binding site for each half. P-gp is located on the apical membrane of epithelial cells. Normally, P-gp is expressed in sensitive organs or tissues such as endothelial cell lining capillaries in the brain, testis, inner ears, pregnant endometrium, placenta, adrenal gland, or even small intestine to prevent these organs or fetus from being exposed to toxic xenobiotics. P-gp is also found to play a role in cellular pharmacokinetics and pharmacodynamics of P-gp substrate anticancer agents. In the intestine, P-gp is found on the apical membrane of enterocytes, but not crypt cells.

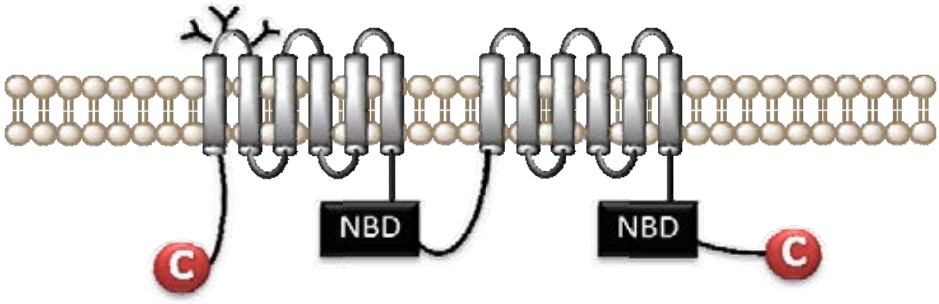


Figure 2: Transmembrane arrangement of P-gp (modified from Sharom, 2008)

3.1 Mechanisms of P-gp efflux (Fardel, Lecureur, and Guillouzo, 1996; Varma et al., 2003)

There are several hypothesized mechanisms for P-gp function. The mechanisms include the classical model, the hydrophobic vacuum cleaner model, and the flippase model as follows.

3.1.1 Classical model

P-gp forms a transmembrane pore. This pore interacts with the substrate in the cytoplasm and excretes it out of the cell.

3.1.2 Hydrophobic vacuum cleaner model

P-gp binds directly with the substrate in the plasma membrane and pumps it out of the cell.

3.1.3 Flippase model

In this model, P-gp encounters the substrate in the inner leaflet of the plasma membrane and flips it to the outer leaflet.

3.2 P-gp substrates (Schinkel and Jonker, 2003, Chan et al., 2004)

Many groups of drugs are reported as substrates of P-gp. Those substrates are not structurally or pharmacologically related. However, there are a few common characteristics for most substrates of P-gp. They are usually organic molecules, ranging in size from less than 200 Da to almost 1900 Da. Many contain aromatic groups, but non-aromatic linear or circular molecules are also classified as substrates of P-gp. Most of P-gp substrates are quite hydrophobic, so they can passively diffuse across cell membranes. Many groups of drugs are classified to be substrates of P-gp, as shown in Table 1.

Table 1: Examples of P-gp substrates (Schinkel and Jonker, 2003, Chan et al., 2004; Palmberger, et al., 2008)

Class	Representative
Analgesics	asimadoline, morphine (poor substrate)
Anticancer drugs	
▪ Vinca alkaloids	vinblastine, vincristine
▪ Taxanes	paclitaxel, docetaxel
▪ Anthracyclines	doxorubicin, daunorubicin, epirubicin
▪ Anthracenes	bisantrene, mitoxantrone
▪ Epipodophyllotoxins	etoposide, teniposide
HIV protease inhibitors	saquinavir, ritonavir, nelfinavir, indinavir, lopinavir, amprenavir, acyclovir
H_2-receptor antagonist	cimetidine
Fluorescent dyes	calcein AM, rhodamine 123, fluo-3

4. Approaches to overcome the function of P-glycoprotein

Several strategies have been used to overcome the function of P-gp. The strategies include synthesis of novel agents that are non-P-gp substrates, the use of P-gp function modulators, and the use of drug delivery systems that allow the drugs to avoid the efflux transporters (Bansal et al., 2009a)

4.1 Synthesis of novel agents that are non-P-gp substrates

Prodrug modification has been introduced to overcome the function of P-gp. In Caco-2 cells, conjugation of propanolol to PAMAM dendrimers can increase the delivery of propanolol in the apical-to-basolateral direction and decrease the transport of the drug in the opposite direction (D'Emanuele et al., 2004). One possible mechanism purposed for this P-gp circumvention is the delivery via endocytosis. However, the use of the cationic dendrimer and the possibility of incomplete conjugation process have raised some concerns regarding the safety issue. Jain and colleagues (2005) have found that prodrug modification by conjugating the P-gp substrate saquinavir with peptides could bypass the function of P-gp. Conjugation with peptides can lead to absorption via peptide transporters. When a substrate binds

to the peptide transporter, it is translocated across the cell membrane and is released into the cytoplasm. During this process, the substrate is not available in the inner leaflet membrane and may avoid recognition by P-gp as a substrate. Prodrug modification of saquinavir, either as Val-Val-saquinavir or as Gly-Val-saquinavir, can enhance the absorption of saquinavir two to five fold. However, peptide transporters are available within a rather narrow window of absorption that can also limit the absorption.

4.2 The use of P-gp function modulators (Krishna and Mayer 2000; Takano et al., 2006)

Numerous studies have employed the substances than can modulate the P-gp function. The co-administration of a P-gp modulator with a P-gp substrate can enhance the intracellular accumulation of the P-gp substrate. A large number of substances have been studied and developed as P-gp function modulators/inhibitors. The chemical structure and the pharmacological action of these modulators are often unrelated. However, there are some structure-activity relationships among the substrates and the modulators/inhibitors of P-gp such as lipophilicity, molecular weight, and molecular chain length. There are some correlations between P-gp inhibition and these characteristics. For example, it is suggested that a highly effective P-gp modulator candidate should possess a log *P* value of 2.92 or higher, 18 atom-long or longer molecular axis, at least one tertiary basic nitrogen atom, and high energy in the highest occupied orbit. The U.S. FDA has introduced the substances that can be used as P-gp inhibitors, as shown in Table 2.

Table 2: Suggested in vitro P-gp inhibitors (Source: U.S. FDA, 2011)

Inhibitor	IC_{50} (μM)	K_i (μM)		
	Caco-2	Caco-2	MDCK-MDR1	LLC-PK1 MDR1
Cyclosporine A	1.3	0.5	2.2	
Ketoconazole	1.2			5.3
LY335979	0.024			
Nelfinavir	1.4			
Quinidine	2.2	3.2	8.6	
Ritonavir	6.5			
Tacrolimus	0.74			
Valspodar (PSC833)	0.11			
Verapamil	2.1	8	15	23
Elacridar (GF120918) (GG918)		0.4	0.4	
Reserpine		1.4	11.5	

Although the use of P-gp inhibitor has been successful in increasing the absorption of P-gp substrates, high doses are usually needed to inhibit adequately the function of P-gp (Bansal et al., 2009b). Because of the high dose used, P-gp inhibitors can lead to undesired intrinsic pharmacological effects or toxic effects as those experienced with cyclosporine A (Ozben, 2006) or verapamil (Varma et al., 2003).

4.3 The use of drug delivery systems

Drug delivery systems that have been used to overcome the function of efflux transporters include micelles, nanoparticles, and liposomes. Micelles and other nanoparticulate drug delivery systems have been used successfully to bypass P-gp function. Dabholkar and coworkers (2006) have reported the use of mixed micelles to increase the accumulation of rhodamine 123, a fluorescent P-gp substrate, in Caco-2 cells. Enhanced accumulation of paclitaxel in the forms of both micelles (Mo et al., 2011) and nanoparticles (Patil et al., 2009) has been reported in in vitro models.

Liposomal drug delivery systems have been extensively used to deliver substrates of P-gp. Liposomal vesicles can entrap both hydrophilic and lipophilic substances. A large number of studies have shown the success of using liposome drug delivery systems to bypass the function of P-gp. The P-gp substrates studied include doxorubicin (Cuvier et al., 1992; Kobayashi et al., 2007; Krieger et al. 2010; Riganti et al., 2011), digoxin (Huwyler et al., 2002), and PSC833 (Bansal et al., 2009a). Though liposomes have been used as drug delivery systems for quite some time, they have been used via the parenteral (Meng et al., 2008; Park et al., 2011) or the transdermal route (Knepp, Szoka, and Guy, 1990; Fang et al., 2006). The success rate of oral delivery via liposomes is limited due to erratic and unpredictable absorption profiles (Ariën et al., 1993). Liposomes are unstable under the gastrointestinal conditions, especially the acidic gastric pH, bile salts, and the pancreatic lipases (Rowland and Woodley, 1980). Upon storage, liposomal preparations also suffer from stability problems such as aggregation, hydrolysis, and oxidation. To overcome these problems, proliposomes have been introduced as alternative drug delivery systems for liposomes (Payne et al., 1986).

5. Proliposome delivery systems

Proliposomes have been defined as dry, free-flowing particles that can form multi-lamella liposomes upon contact with the aqueous phase (Payne et al., 1986). Early studies have revealed that proliposome delivery could improve the oral absorption of several substances including exemestane (Hiremath et al., 2009), silymarin (Yan-yu et al., 2006), vinpocetine (Xu et al., 2009), and cromolyn sodium (Deshmukh et al., 2008). However, there is no study that directly assesses the use of proliposomes to improve the oral absorption of P-gp substrates.

Several studies have investigated the use of proliposome delivery systems as oral dosage forms. By improving solubility, proliposomes have been used to deliver exemestane, an oral steroidal aromatase inactivator, in rat intestine, Caco-2 cells, and parallel artificial membranes models. These results showed that proliposome delivery system could enhance the dissolution and the permeability of exemestane, and thus its absorption in all of those models (Hiremath et al., 2009). The advantages of proliposomes have also been revealed in vivo. Yan-yu et al. (2006) have prepared

silymarin proliposomes to increase oral bioavailability of the drug in beagle dogs. Silymarin shows the poor solubility in both water and in oils, as well as poor permeation across intestinal epithelial membranes. The result showed that silymarin proliposomes could increase the absorbed amount of silymarin up to 3.4 fold when compared to the solution form. Furthermore, the use of proliposomes to improve the delivery of vinpocetine in New Zealand rabbits was also successful (Xu et al., 2009). Moreover, proliposome delivery systems have been used to deliver water soluble substances to improve their permeability. Proliposome beads of cromolyn sodium successfully enhanced the delivery of cromolyn across Caco-2 monolayers and the intestinal membrane (Deshmukh, et al., 2008).

Since proliposomes are a solid dosage form, it can be applied to many administration routes. Proliposomes have also been used to deliver nicotine by the transdermal route. Nicotine-containing proliposomes have been delivered through abdominal skin of rat under an occlusive condition. The result showed that nicotine-containing proliposomes gave a constant flux when compared to nicotine powders, which could be used for sustained release purposes (Hwang et al., 1997). In addition, clotrimazole-loaded proliposomes successfully enhanced and prolonged delivery of clotrimazole in vaginal tissue without causing inflammation or necrosis of the tissue (Ning et al., 2005). Moreover, proliposomes for intranasal delivery have been studied. Propranolol-containing proliposomes successfully delivered propanolol to blood circulation by intranasal administration with the abosolute bioavailability of 103.1% (Ahn, Kim, and Shim, 1995).

Similar to liposome delivery systems, proliposome delivery systems can provide sustained release of drugs. Several studies have shown the prolonged or sustained release of drugs from proliposomes (Ahn et al., 1995; Hwang et al., 1997; Jung et al., 2000a; Junping et al., 2000; Ning et al., 2005).

There are several methods for preparation of proliposomes. These include the crystal-film method, the film-deposition-on-carriers method, the freezing and drying method, the powder-bed grinding method, the spray drying method, and the supercritical anti-solvent method (Junping et al., 2000; Song, et al., 2002; Yan-yu et al., 2006; Deshmukh et al.,2008; Xia et al., 2010). However, the method used in most

of proliposome research has been the film-deposition-on-carriers method since the method is the most practical for laboratory scales.

In the method of film deposition on carriers, the solution of phospholipid in organic solvent is added onto the surface of the carriers. Proliposomes are obtained after all the organic solvent is evaporated and the phospholipid deposits onto the carriers.

Various materials have been evaluated for their suitability to use as the carriers. These materials include glucose, fructose, lactose, sodium chloride, sorbitol, and mannitol (Payne et al., 1986; Yan-yu et al., 2006). Above all other materials, sorbitol shows the desired characteristics. Sorbitol possesses good water solubility and poor chloroform and/or methanol solubility. Though mannitol is also widely used in the pharmaceutical field, sorbitol provides better water solubility than mannitol (Rowe, Sheskey, and Owen, 2006). Most of proliposomes prepared by film deposition on carriers used sorbitol as the carriers (Kartare, Vyas, and Dixit, 1991; Ahn et al., 1995; Hwang et al., 1997; Ning et al, 2005; Song et al., 2005).

Sorbitol particles that are suitable for proliposome preparation should have particle sizes ranging from 125 to 500 μm (Payne et al., 1986). The sorbitol particles in the range of 125-500 μm provide the characteristics suitable for proliposome preparation when a constant weight ratio of liposome components to sorbitol is maintained. Besides, sorbitol particles in this size range do not introduce any effects on subsequent liposome size (Payne et al., 1986).

6. Caco-2 cells as in vitro models (Bailey, Bryla, and Malick, 1996; Shah et al., 2006)

Caco-2 cells are derived from a human colonic adenocarcinoma. After spontaneous differentiation, Caco-2 cells display morphological and functional characteristics similar to those of the intestinal enterocytes. In addition, the cells form tight junctions and express brush border enzymes, some cytocrome P isoenzymes, and phase II enzymes such as glutathiones-*S*-transferases, sulfotransferases, and glucoronidases.

Caco-2 cells also provide the absorption mechanisms available in enterocytes including passive transcellular and paracellular, carrier-mediated influx and efflux, and vesicular transport pathways. Many studies have revealed the correlation between the permeability across Caco-2 cells and in vivo absorption. Thus, Caco-2 cells have been extensively used to study the absorption of compounds with diversified physicochemical properties. Among these are cromolyn sodium (Deshmukh et al., 2008), exemestane (Hiremath et al., 2009), and cephalexin (Gochoco et al., 1994).

Besides drug absorption, Caco-2 cells have been used to evaluate the cytotoxicity, drug–drug/drug–excipient interaction, and metabolic stability. Similar to other carcinoma cell lines, Caco-2 cells exhibit several ATPase-dependent drug efflux pumps such as P-gp, MRP, and breast cancer resistant protein (BCRP) (Takano et al., 2006). Many studies have also utilized Caco-2 cells to investigate the effect of P-gp on the uptake or transport of P-gp substrates including vinblastine (Hunter, Hirst, and Simmons, 1993), indinavir (Hochman et al., 2001), clopidogrel (Taubert et al., 2006), and irrinotecan (Bansal et al., 2009b).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Acetic acid, glacial (Merck, Germany, Lot no. K33266463422)
2. Acyclovir micronized (Zhejiang Charioteer Pharmaceutical Co., Ltd., People's Republic of China, Batch no. A090122-212)
3. Bradford's reagent (Sigma, USA, Batch no. 080M4359)
4. Calcein (Sigma, USA, Lot no. 020M1241)
5. Calcein acetoxymethyl ester (Fluka, Switzerland, Lot no. CBC4107)
6. Chloroform, AR grade (RCI-Labscan, Thailand, Batch no. 08041093)
7. Cholesterol (Fluka, Japan, Lot no. 1324049)
8. Cyclosporine A (a gift from Dr. Suree Jianmongkol, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University)
9. Dimetyl sulphoxide (DMSO) (Sigma, USA, Lot no. 076K2321)
10. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA, Lot nos. 866169 and 984295)
11. D-sorbitol (Unilab, Australia**,** Batch no. 903225**)**
12. Eagle's Minimal Essential Medium (MEM) non-essential amino acids (Gibco, USA, Lot nos. 787187, 889721, 901749, and 988726)
13. Fetal bovine serum (Biochrom AG, Germany, Lot nos. 0690T, 1376W, and 0247X)
14. HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, USA, Batch no. 040M5424)
15. L-Glutamine (Gibco, USA, Lot nos. 802013, 872417, and 915597)
16. MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan thiazoyl blue formazan) (Sigma, USA, Lot no. 094K5312)
17. Penicillin-Streptomycin (Gibco, USA, Lot nos. 793252, 861670, 907168, and 918577)
18. Rhodamine 123 (Sigma, USA, Lot no. 017K3696)
19. SL-2 Sigmacote® (Sigma, USA, Lot no. 078K4370)

20. Sodium bicarbonate (Srichand United Dispensary, Co., Ltd., Thailand, Lot no. 1002030019)
21. Sodium chloride (Merck, Germany, Lot no. K38447104807)
22. Sodium hydroxide (Merck, Germany, Lot no. B0200898803)
23. Soybean phosphatidylcholine (Phospholipon 90® Nattemann Phospholipid, GmbH, Cologne, Germany, Lot no. 770991)
24. Triton X-100 (Sigma, USA, Lot no. 40F1535)
25. Trypan blue (Sigma, USA, Lot no. 87F50385)
26. Trypsin-EDTA (Gibco, USA, Lot nos. 822908, 833117, and 930004)
27. α-Tocopherol (Approx. 95%, Sigma, USA, Lot no. 063K0796)
28. ±Verapamil hydrochoride (Sigma, USA, Lot no. 021M1429V)

Equipment

1. Analytical balances (AX105, PG403-S, and UMT2, Metler Toledo, Switzerland)
2. Autoclave (Hirayama, Japan)
3. Centrifuge (Minispin®, Eppendorf, Germany)
4. Hand-held extruder (LiposoFast™, AVESTIN, Canada)
5. High performance liquid chromatography (LC-10, Shimadzu, Japan; 1100 series, Agilent, Germany)
6. Hot air oven (MEMMERT, Germany)
7. Humidified carbon dioxide incubator (Thermo Electron Corporation, USA)
8. Incubator (Precision, USA)
9. Laminar air-flow hood (Microflow, UK)
10. Light microscope (CKX41, IX51, Olympus; Eclipse E200, Nikon, Japan)
11. Lyophilizer (Dura-Dry™ Microprocessor, USA)
12. Microplate reader (VICTOR3, Perkin Elmer, USA)
13. Orbital shaker (SO5, Bibby, Stuart Scientific, UK)
14. Osmometer (Osmomat® 030D, Gonotec GmbH, Germany)
15. pH meter (PB 20, Sartorius, USA)
16. Rotary evaporator (R 215, Buchi, Switzerland)
17. Ultracentrifuge (Beckman L-80, USA)

18. UV spectrophotometer (Model UV-1601, Shimadzu, Japan)
19. Vortex mixer (G-560E, Scientific Industries, USA)

Laboratory supplies

1. Nylon membrane filter (0.45 µm) (Vertical Chromatography Co., Ltd., Thailand)
2. Multiwell plates (Corning, USA)
3. Polycarbonate membrane (100 nm) (Millipore®, USA)
4. Sterilization filtration membranes (0.22 µm) (Pall Corporation, USA)
5. Tissue culture flasks (Corning, USA)

Cell line

Caco-2 cells (American Type Culture Collection, ATCC®: HTB-37™)

Methods

1. Preparation and characterization of proliposomes

1.1. Determination of maximum phospholipid loading

The aim of this experiment was to determine the amount of soybean phosphatidylcholine (SPC) that could be loaded onto a fixed amount of the sorbitol carrier without significant changes in the physical properties of the carrier particles. Blank proliposomes were prepared by film deposition on carriers, adapted from the work of Song et al. (2002). The equipment was modified and set as depicted in Figure 3.

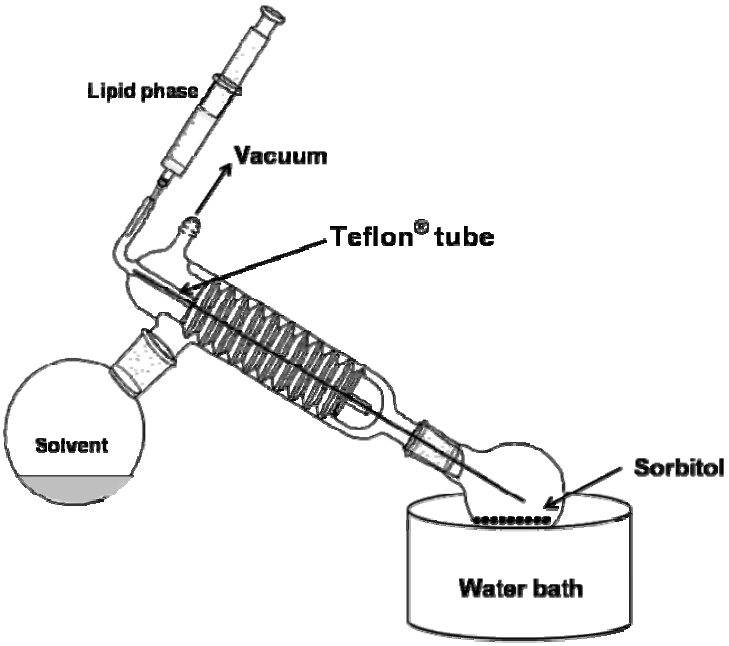


Figure3: The modified rotary evaporator for preparation of proliposomes (modified from Song et al., 2002)

The preparation method for proliposomes in this study was as follows. A portion of sorbitol particles was placed in a round bottom flask assembled to the rotary evaporator. The water bath was kept at 45°C. The rotation rate was adjusted to 40 rpm. SPC was dissolved in chloroform at a concentration of 75 mg/ml. The lipid solution was added onto the sorbitol particles in the round bottom flask from the syringe reservoir at the top of the condenser via a Teflon® tube. The lipid drop rate was strictly controlled using the vacuum valve in order not to inundate the sorbitol carrier. The process was continued until all of the solution was introduced. The rotary evaporator was running until free flowing particles were obtained. To ascertain that no trace lipid was left in the tubing, the tubing was flushed with a small amount of chloroform. After all of chloroform was evaporated, proliposomes were obtained. The whole preparation process took about 2 hours, during which the syringe reservoir was covered with aluminum foil.

This study was conducted in the following steps:

1.1.1. Preparation of blank proliposomes with various amounts of SPC

Proliposomes were prepared by the modified film-deposition-on-carriers method as described above. Sorbitol with the particle size ranging from 125 to 500 μm has been reported to be suitable for proliposome preparation (Payne et al., 1986). In this present study, sorbitol with particle size less than 425 μm was used. The majority (≥ 75%) of these sorbitol particles was within the size range of 150-425 μm as measured by sieving (see Appendix A for particle sizes). The amount of SPC was varied from 0.18-0.35 mmol per one gram of sorbitol. The starting SPC amount used (0.35 mmol/g) was estimated from the surface area of sorbitol and the lipid loading previously reported (total pore area of sorbitol = 33.1 m^2/g, lipid loading = 7.9 mg/m^2) (Payne et al, 1986). Blank proliposome preparations containing 0.35, 0.31, 0.26, 0.22, and 0.18 mmol of SPC/g of sorbitol were prepared. The physical properties of these preparations were compared with those of sorbitol. The maximum lipid loading without major changes in surface morphology and without compromising the flow properties of the particles was selected for further experiments.

1.1.2. Physical properties of the blank proliposomes

An excess of phospholipid on the surface of proliposomes can cause particle agglomeration and compromise the flow properties of the particles (Payne et al., 1986). Scanning electron microscopy was used to monitor surface morphology of proliposomes for excesses of phospholipid (Annakula et al., 2010). The flow properties were evaluated by means of the bulk density and the angle of repose (Annakula et al., 2010; Gustavo, 2005).

1.1.2.1. Investigation of surface morphology of proliposomes by scanning electron microscopy

The surface of blank proliposome particles was fixed onto a scanning electron microscope stub and observed under a scanning electron microscope at 15 kV without further coating with any metals. Sorbitol particles were used for comparison. The proliposome formulations with the surface morphology close to that of sorbitol were selected as candidates. This property was considered along with the flow properties for further formulation selection.

1.1.2.2. Determination of the angle of repose (The United States Pharmacopeia Convention, 2012)

The angle of repose of all blank proliposome formulations was determined and categorized according to the United States Pharmacopeia (The United States Pharmacopeia Convention, 2012) along with that of the sorbitol particles. Briefly, proliposome particles were filled into a funnel fixed at a suitable height. The suitable height was determined from preliminary runs. The funnel height was maintained at approximately 3 cm from the top of the pile of proliposomes. An aliquot (4-5 g) of proliposome particles was drained from the funnel to a fixed diameter base. The height of the symmetric cone of the proliposome particles was measured. The angle of repose was calculated from the following equation:

$$\tan\theta = \frac{\text{height}}{0.5\ \text{base}}$$

1.1.2.3. Determination of bulk density (The United States Pharmacopeia Convention, 2012)

The bulk density was measured by a recommended procedure according to the United States Pharmacopeia (The United States Pharmacopeia Convention, 2012). The bulk density of sorbitol was used for comparison. To determine the bulk density, blank proliposomes (approximately 4-5 g) was gently filled into a 25 ml cylinder. The volume of blank proliposomes was measured. The bulk density of blank proliposomes was calculated from the following equation:

$$\text{Bulk density} = \frac{\text{weight of blank proliposomes (g)}}{\text{volume of blank proliposomes (cm}^3\text{)}}$$

Blank proliposome formulations that could accommodate the highest amount of SPC without any evidence of excess SPC and with acceptable flow properties were selected. A further titration for a more optimized lipid amount was carried out in a similar manner. The selected blank formulation was hydrated with deionized water at 37°C to corroborate the formation of liposomes from proliposomes upon contact with an aqueous phase.

1.2. Determination of SPC to cholesterol (CHO) ratio

The purpose of this study was to determine a suitable ratio of SPC and CHO that could be loaded onto the sorbitol carrier. CHO is known to improve the stability of liposomes (Kirby, Clarke, and Gregoriadis, 1980). CHO molecules can be inserted into the lipid bilayer, and thus affect the membrane permeability. In case of liposomes containing hydrophilic molecules, CHO can prevent the leakage of hydrophilic molecules from liposome vesicles (Kang et al., 2009).

In this study, the amount of SPC from the previous study was used as the amount of total lipid for preparation of proliposomes. Proliposomes different in the SPC:CHO ratio (1:0, 1:0.25, 1:0.5, and 1:1) were prepared as previously described. In brief, both SPC and CHO were dissolved in chloroform. The mixed lipid solution was allowed to deposit onto the sorbitol carrier as described in Section 1.1. The physical properties of the resultant proliposomes with various SPC:CHO ratios were evaluated as described in Section 1.1.2.

All formulations were separately hydrated with Ultrapure® water at 37°C. Formulations resulting in complete formation of liposome dispersion were selected for further preparation of P-gp substrate-loaded proliposomes.

1.3. Preparation of P-gp substrate-loaded proliposomes

1.3.1. Preparation of calcein AM-loaded proliposomes

Calcein AM-loaded proliposomes were prepared by the film-deposition-on-carriers method. Calcein AM (11.9 nmol/g of sorbitol) was dissolved in the chloroform containing SPC, CHO, and 0.1 mol% α-tocopherol. The lipid solution was added dropwise onto the carrier in a 100 ml round bottom flask via a Teflon® tube, as described in Section 1.1. After all chloroform was evaporated, calcein AM-loaded proliposomes were obtained. Calcein AM is extremely susceptible to moisture, leading to decomposition of the dye (Molecular Probes®, 2010). The calcein AM-containing proliposomes were freshly prepared and used within 24 hours for the uptake study. Thus, stability of calcein AM-loaded proliposomes was not evaluated.

The amount of calcein AM was selected such that, upon hydration and further dilution of proliposome preparations, the concentration of calcein AM would be 25 nM and the total lipid would be 0.46 mM in the uptake study. These concentrations

were chosen based on a previous study on calcein AM liposomes in this laboratory (Ing-orn Prasanchaimontri, 2009).

1.3.2. Preparation of acyclovir-loaded proliposomes

To ascertain that acyclovir would distribute evenly throughout the carrier, acyclovir (0.43 mmol/g of sorbitol) and sorbitol were dissolved in Ultrapure® water and lyophilized until a dry matrix was obtained. The process was done under a light protection protocol and usually took around 48-72 hours. The dry matrix was gently ground into fine particles. The resultant particles were sieved through the 425 µm-opening sieves. Particle size distribution was determined by sieving and compared to that of sorbitol. The particles that passed through the 425 µm-opening sieves were used as the carrier for the preparation of acyclovir proliposomes. The proliposome preparations with various CHO contents selected from Section 1.2 were prepared as described in Section 1.1.

1.4 Characterization of P-gp substrate-loaded proliposomes

P-gp substrate-loaded proliposomes were characterized for angles of repose and bulk densities as described in Sections 1.1.2.2 and 1.1.2.3, respectively.

1.5 Stability of acyclovir-loaded proliposomes

The preliminary study showed that liposomal entrapment of acyclovir prepared from proliposomes decreased with proliposome storage time. A reduction in liposome entrapment of more than 20% occurred within one month upon storage of proliposomes. The purpose of this study was to evaluate how long acyclovir-loaded proliposomes could be kept under the storage condition before being used in the uptake study. Acyclovir-loaded proliposomes were prepared as described in Section 1.3.2. The addition of 0.1 mol% of α-tocopherol was also used for stability improvement. The resultant proliposomes were flushed with nitrogen gas and kept in a desiccator at -20°C. The entrapment efficiency of liposomes prepared from acyclovir-loaded proliposomes was determined on the day of proliposome preparation and at 7 days and 14 days of storage.

For entrapment efficiency determination, acyclovir-loaded proliposomes were hydrated in a modified simulated intestinal fluid (mSIF) (see Appendix B) at 37°C on an orbital shaker, at 100 rpm, for 60 minutes to give the total lipid concentration and acyclovir concentration of 0.35 mg/ml and 250 µg/ml (0.46 mM and 1 mM),

respectively. The resultant liposome suspension was centrifuged at 50,000 rpm, 25°C for 3 hours to separate liposomes from the aqueous phase. Both the supernatant and the pellet were assayed for the amount of SPC and acyclovir. SPC was determined by the Bartlett assay (New, 1989) (Appendix C). Potassium dihydrogen phosphate was used to construct standard calibration lines for SPC determination. Acyclovir was determined by the validated UV spectrophotometric method at 254 nm (Appendix D). The entrapment efficiency of acyclovir-loaded proliposomes was calculated from the following equation.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Acyclovir amount in pellet (mg)}}{\text{Total lipid amount in pellet (mg)}} \text{ x } 100$$

2. Maintenance of Caco-2 cells

Caco-2 cells were cultivated in a humidified CO_2 incubator at 37°C. Cells were grown in the complete culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 1mM sodium pyruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin, 1% MEM non-essential amino acid, and 10% fetal bovine serum. Caco-2 cells were subcultured at 70-80% confluence. Briefly, the cell monolayer was washed with 10 ml of phosphate buffered saline (PBS). Trypsin (0.25%) in 1 mM EDTA solution (trypsin-EDTA) was added (1.2 ml/75 cm^2) to the culture flask and the flask was further incubated for 3-4 minutes at 37°C. An aliquot (5 ml) of the complete medium was added to inactivate trypsin. The content of the flask was thoroughly triturated to disperse the cells. The dispersed cells were centrifuged and the supernatant was removed. Cells were re-dispersed with the complete medium and seeded into a new 75 cm^2 flask with a cell density of 8-9 x 10^5 cells per 15 ml. The medium was replaced with the fresh one at 24 hours after seeding and then every other day until the next subculture.

For the uptake experiments, cells were grown in culture flasks and harvested as stated above. The cell suspension was seeded into 24-well culture plates at a concentration of 2.5×10^4 cells/well. The cell monolayer was grown for 21 days with

the medium changed every other day. The medium was also changed at 12 hours before an experiment was carried out.

3. Effect of the mSIF medium on P-gp function

The aim of this study was to examine whether the mSIF medium would affect the P-gp function since HEPES is known to increase P-gp function by increasing the ATPase activity (Luo et al., 2010). In this study, the uptake of a fluorescent P-gp substrate, rhodamine 123, with and without the P-gp inhibitor verapamil, in DMEM and in mSIF was monitored by spectrofluorometry. The extents of rhodamine 123 associated with the cells were calculated and compared. Briefly, Caco-2 cells were washed with pre-warmed PBS (1 ml/well). The DMEM or mSIF solution, with or without 100 µM verapamil, was added and the cells were further incubated at 37°C for 30 minutes. After the pretreatment period, the solution was removed. The DMEM or the mSIF solution containing 2 µM of rhodamine 123 (with and without 100 µM verapamil) was then added to the cells in each respective well. The cells were further incubated for 120 minutes. At the end of the incubation, cells were washed four times with ice-cold PBS (0.5 ml/well). Cells were lyzed with 1% Triton X-100 in PBS for 120 minutes at 37°C on an orbital shaker rotating at 75 rpm. Rhodamine 123 solution was protected from light at all times and the culture plates were wrapped with aluminum foil during the experiment. Cell-associated rhodamine 123 was detected using a microplate reader at the excitation/emission wavelengths of 485/535 nm. Rhodamine 123 concentrations were back calculated from the standard calibration line prepared for each experimental run (Appendix E).

4. Selection of the uptake medium

Since mSIF affected the function of P-gp, the uptake medium was modified by mixing mSIF with DMEM at various ratios. This study was undertaken to find a suitable ratio of mSIF and DMEM that could be used as the uptake medium without or least affecting the P-gp function. The ratio of mSIF:DMEM was varied to be 0:100, 25:75, 50:50, and 75:25. The osmolality of the medium was determined by osmometer (Osmomat® 030D, Gonotec GmbH, Germany). Rhodamine 123 (2 µM) and verapamil (100 µM) was used as the P-gp substrate and the inhibitor, respectively. The uptake experiment was conducted as described in Section 3. The

reverse ratios of rhodamine 123 for the tested uptake media were compared. The reverse ratio was defined as the ratio between the concentrations of P-gp substrate accumulated in the cells in the presence and in the absence of the inhibitor.

The suitable ratio of mSIF:DMEM obtained from this experiment was further used as the uptake medium for all following experiments.

5. Verification of Caco-2 cells

5.1 Verification of P-gp function

The purpose of this study was to verify that Caco-2 cells showed P-gp function under the culture condition. This verification was routinely done for all relevant experiments. The uptake of either calcein AM or rhodamine 123, both are known P-gp substrates, in the absence and the presence of a P-gp inhibitor (verapamil or cyclosporine A) was evaluated.

The uptake experiment was conducted in a similar manner to the method described in Section 3. Briefly, Caco-2 cells were allowed to grow in 24-well culture plates for 21 days. The medium was changed at 12 hours before the experiment was performed. On the experiment day, Caco-2 cells were washed with 1 ml pre-warmed PBS. After washing, cell monolayer was pre-incubated with 0.5 ml of the uptake medium with and without a P-gp inhibitor (100 µM verapamil or 5 µM cyclosporine A) for 30 minutes.

When calcein AM was used, the cells were incubated with 25 nM calcein AM with or without a P-gp inhibitor for 90 minutes. Caco-2 cells were washed four times with ice-cold PBS. The cells were dissolved with 0.3 N sodium hydroxide solution containing 1% Triton X-100 at 37°C on an orbital shaker, at 75 rpm, for 120 minutes. After cell lysis, a clear solution was obtained. The clear solution was assayed for the fluorescent intensity at the excitation wavelength of 485 nm and the emission wavelength of 535 nm to determine the concentration of calcein accumulated in the cells. The calcein standard calibration line, constructed in the presence of the cells as the background (Appendix E), was used to calculate the calcein concentration. The reverse ratio was calculated accordingly. For rhodamine 123 uptake, cells were incubated with 2 µM of rhodamine 123 instead of calcein AM. The experiment was carried out in a similar manner to that of calcein AM, with the incubation time of 120

minutes. The lyzing solution for rhodamine 123 uptake was 1% Triton X-100 in PBS, pH 7.4. Control cells were not included as the background in the standard calibration line for rhodamine 123 since the interference from the cells was minimal (Appendix F).

5.2 Verification of enhanced accumulation of fluorescent markers in Caco-2 cells via liposomal delivery

The enhanced cellular uptake of P-gp substrates using proliposome delivery systems relies upon the ability of the cells to take up the resultant liposomes. To verify that Caco-2 cells were capable of liposome uptake, calcein and calcein AM were used as markers (Araya Lukanawonakul, 2005; Ing-orn Prasanchaimontri, 2009). Calcein is a hydrophilic fluorescent dye widely used in liposome research to demonstrate the advantage of liposomes for the delivery of water-soluble compounds. Calcein AM, on the other hand, is a relatively lipophilic compound and is often used as a marker for P-gp substrates. In this set of experiments, uptake of calcein-loaded liposomes at various concentrations was evaluated and compared with that of calcein solution. In addition, most of the studies evaluating cellular uptake of liposomes were usually done using liposomes with controlled size (Boman et al., 1994; Lo et al., 2001; Chang et al., 2011). However, liposomes generated from proliposomes are usually heterogeneous in size (Song et al., 2005; Deshmukh et al., 2008). Hence, the ability of the Caco-2 cells to uptake liposomes with different size distribution profiles was also investigated using calcein AM as a fluorescent marker.

5.2.1 Preparation of calcein-loaded liposomes (New, 1989; Araya Lukanawonakul, 2005)

Calcein-loaded liposomes were prepared by the conventional film hydration method with the total lipid concentration of 50 mg/ml. The liposome composition comprised SPC and CHO at the molar ratio 7:3. Alpha-tocopherol (0.1 mol %) was used as an antioxidant in all preparations. Briefly, the lipid components were dissolved in chloroform in a round bottom flask attached to a rotary evaporator. Chloroform in the round bottom flask was evaporated at a controlled rate, resulting in a thin film of mixed lipid. The thin film was kept under vacuum for at least 2 hours to eliminate all traces of the organic solvent. After all of the organic solvent was evaporated, the film was hydrated with the aqueous phase containing 80 mM of

calcein in 0.3 N sodium hydroxide. The resultant liposomes were monitored under a light microscope. The liposomal preparation was further diluted with the uptake medium to the desired concentrations. The calcein-loaded liposomes were freshly prepared and used within 24 hours.

5.2.2 Effect of liposome concentration on the accumulation of calcein in Caco-2 cells (Araya Lukanawonakul, 2005; Ing-orn Prasanchaimontri, 2009)

The uptake experiment was performed in a similar manner to the experiments in Section 3. Calcein solutions at comparable concentrations were used as controls. Briefly, Caco-2 cells were pre-incubated with the uptake medium at 37°C for 30 minutes. After the pre-incubation period, the uptake medium was removed. Caco-2 cells were further incubated with either calcein solution or calcein-loaded liposomes for 120 minutes. The concentrations of calcein used in this experiment were 20, 80, and 140 µM for both calcein solutions and calcein-loaded liposome preparations. At the end of the incubation time, Caco-2 cells were washed 4 times with ice-cold PBS (0.5 ml/well). Caco-2 cells were lyzed with 0.3 N sodium hydroxide containing 1% Triton X-100. The resultant solution was further assayed for calcein contents. Calcein in 0.3 N sodium hydroxide containing 1% Triton X-100 at various concentrations was used to construct the standard calibration line. The extents of calcein accumulation in Caco-2 cells from calcein solution and calcein-loaded liposomes were compared.

5.2.3 Preparation of calcein AM-loaded liposomes

Calcein AM-loaded liposome preparation was prepared by the conventional film hydration method. Alpha-tocopherol (0.1 mol %) was used as antioxidant in the preparation. Briefly, SPC (35 mg/ml) and calcein AM (2.5 nmol/ml) were dissolved in chloroform in a round bottom flask attached to the rotary evaporator. Chloroform in the round bottom flask was evaporated, resulting in thin film of mixture of SPC and calcein AM. The thin film was kept under vacuum for at least 2 hours to eliminate all traces of the organic solvent. After all of the organic solvent was evaporated, the film was hydrated with the mSIF as the aqueous phase. The liposomes were examined routinely under a light microscope. The calcein AM-loaded liposomes were extruded through a 100 nm polycarbonate membrane to reduce the liposome size to approximately 100 nm.

5.2.4 Effect of size distribution profile on the accumulation of calcein AM in Caco-2 cells

The uptake experiment was carried out in a similar manner to the experiments in Section 3. The uptake of calcein AM from the calcein AM solution, the calcein AM-loaded liposomes, and the extruded calcein AM-loaded liposome was compared. Briefly, Caco-2 cells were pre-incubated with the uptake medium at 37°C for 30 minutes. After the pre-incubation period, the uptake medium was removed. Caco-2 cells were further incubated with the calcein AM solution, the calcein AM-loaded liposomes or the extruded calcein AM-loaded liposomes for 90 minutes. The concentration of calcein AM used in this experiment was 25 nM. At the end of the incubation time, Caco-2 cells were washed 4 times with ice-cold PBS (0.5 ml/well). Caco-2 cells were lyzed with 0.3 N sodium hydroxide containing 1% Triton X-100. The resultant solution was further assayed for extents of calcein accumulated in Caco-2 cells. Calcein in 0.3 N sodium hydroxide containing 1% Triton X-100 at various concentrations was used to construct the standard calibration line. The extents of calcein accumulation in Caco-2 cells from the calcein AM solution, the calcein AM-loaded liposomes, and the extruded calcein AM-loaded liposome were compared.

6. Effects of proliposome systems on the accumulation of P-gp substrates in Caco-2 cells

The objective of this study was to assess whether the selected proliposome systems could enhance the intracellular accumulation of the model P-gp substrates, calcein AM and acyclovir. The solution of either calcein AM or acyclovir was used as the control. The results from the preliminary experiment showed that all formulations used in the uptake experiments did not affect the viability of Caco-2 cells. The cell viability was higher than 90% of the control in all cases (see Appendix G).

6.1 Uptake of calcein AM from proliposome systems

For calcein AM, the experiment was carried out by comparing the uptake of the calcein AM solution, the extruded calcein AM-loaded liposomes, and the calcein AM-loaded proliposomes, with and without CHO.

Calcein AM-loaded liposomes were prepared by the conventional film-hydration method followed by extrusion, as described in Section 5.2.3, at the total

lipid of 35 mg/ml. The calcein AM-loaded proliposome preparations were pre-hydrated in the mSIF, pH 6.8, at 37°C with continuous shaking at 75 rpm on an orbital shaker for 60 minutes. Homogeneous dispersions of calcein AM-loaded liposomes were generated.

In the uptake experiment, the extruded calcein AM-loaded liposome preparation was diluted with the uptake medium to yield 0.35 mg/ml of lipid. Calcein AM-loaded proliposomes was hydrated with mSIF and further diluted with DMEM to yield the comparable concentrations of the lipid and calcein AM in the uptake medium. This resulted in the test formulations being in the same uptake medium. The total lipid concentration of all liposome preparations was 0.35 mg/ml. The concentration of calcein AM was 25 nM. The uptake experiments were carried out as described in Section 3.

6.2 Uptake of acyclovir from proliposome systems

The experiment was conducted in a similar manner to that of calcein AM-loaded proliposomes. The acyclovir solution, the extruded acyclovir-loaded liposomes, and the two proliposome formulations (with and without CHO) were compared. The total lipid concentration of the extruded acyclovir-loaded liposomes and the proliposome formulations was 0.35 mg/ml. The concentration of acyclovir used in the uptake experiment was 250 µg /ml (ca 1 mM) (Palmberger et al., 2008). Acyclovir-loaded liposomes were also prepared by the conventional film hydration method at 0.7 mg/ml of total lipid. Briefly, the thin film of SPC was prepared. The solution of 2mM acyclovir in mSIF was added to hydrate the film, resulting in acyclovir-loaded liposomes. In the uptake experiment, the extruded acyclovir-loaded liposome preparation was diluted with an equal amount of DMEM to yield 0.35 mg/ml of total lipid in the uptake medium. The acyclovir proliposome preparations were hydrated with mSIF and further diluted with DMEM to yield the required lipid and acyclovir concentrations in the uptake medium. The uptake experiment was carried out as described in Section 3 for 120 minutes. At the end of the incubation time, Caco-2 cells were washed four times with ice-cold PBS and lyzed with 1% Triton X-100 in PBS. The resultant mixture was centrifuged at 13000 rpm (Minispin®, Eppendorf, Germany) for 5 minutes. The supernatant was filtered through 0.45 µm Nylon membrane filters. The concentration of acyclovir in the supernatant

was determined by the verified HPLC method with a UV detector (see Appendix H). The standard calibration line of acyclovir was constructed for each analytical run. The extents of acyclovir associated with the cells from the 4 treatments were compared.

7. Statistical analysis

The experiments were performed at least in triplicate. The data were presented as means ± SEM (standard error of mean) or means ± SD (standard deviation). If the data showed the homogeneity of variances, the analysis of variance (ANOVA) was used at $\alpha = 0.05$ to compare the treatment means. The Scheffe's test was used as the post hoc multiple comparison test. Otherwise, Welch's ANOVA was used at $\alpha = 0.05$ to compare the treatment means followed by Dunnett's T3 for the post hoc test (Kinnear and Gray, 2011). The statistical analysis was performed on the SPSS Statistics Base 17.0 for Windows (SPSS serial no.: 5068054).

CHAPTER IV

RESULTS AND DISCUSSION

1. Preparation and characterization of proliposomes

1.1. Determination of maximum phospholipid loading onto the sorbitol carrier

The physical appearance and the surface morphology of blank proliposomes at various phospholipid:sorbitol ratios are shown in Figures 4 and 5, respectively.

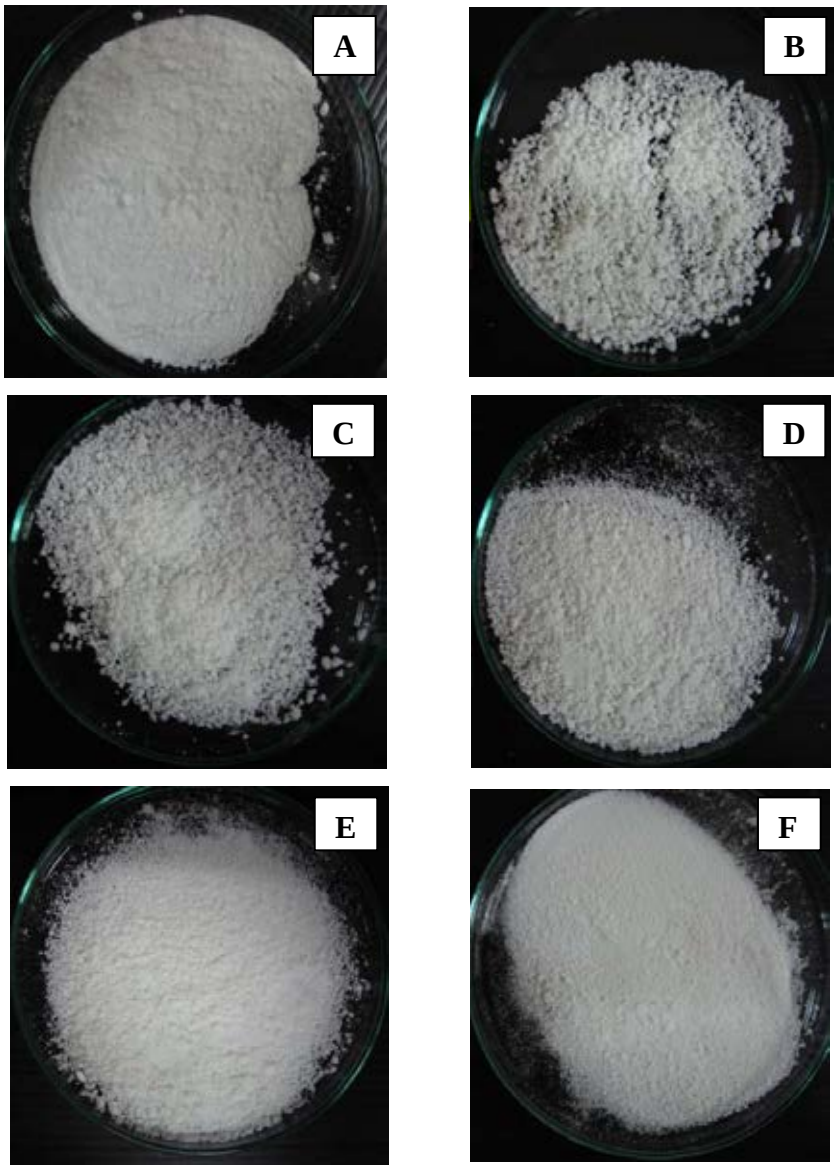


Figure 4: Physical appearances of (A) sorbitol particles and blank proliposomes containing (B) 0.35, (C) 0.31, (D) 0.26, (E) 0.22, and (F) 0.18 mmol of SPC/g of sorbitol

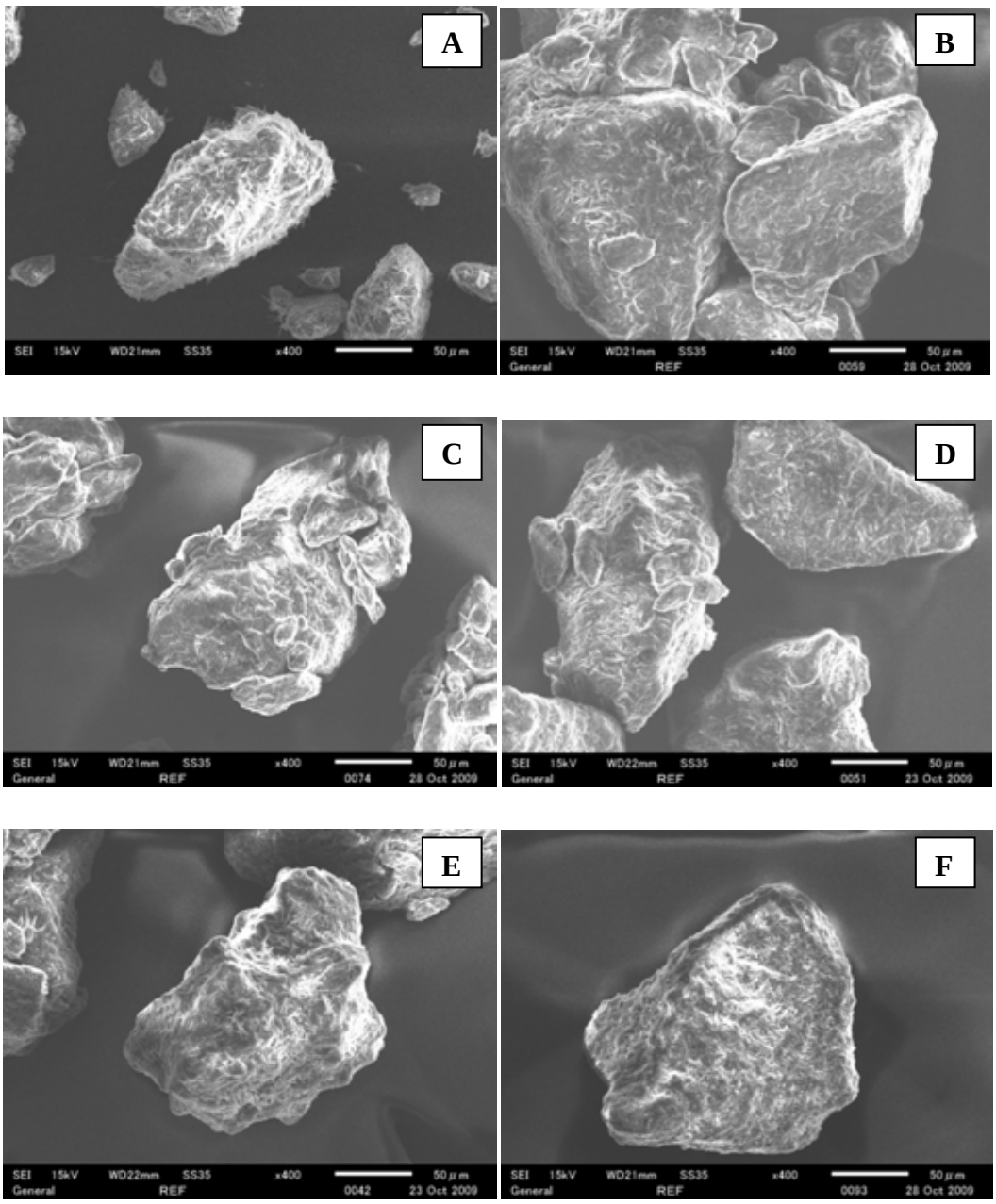


Figure 5: Electron micrographs of (A) sorbitol particles and blank proliposomes containing (B) 0.35, (C) 0.31, (D) 0.26, (E) 0.22, and (F) 0.18 mmol of SPC/g of sorbitol

Blank proliposomes prepared with 0.35, 0.31, and 0.26 mmol of SPC/g of sorbitol resulted in the excess SPC (Figure 4, B-D). The excess SPC on the sorbitol particles caused the agglomeration of the particles. On the other hand, the appearance of blank proliposomes prepared with 0.22 and 0.18 mmol of SPC/g of sorbitol was comparatively similar to that of sorbitol particles (Figure 4, E-F).

The surface morphology of proliposomes containing 0.35 and 0.31 mmol of SPC/g of sorbitol (Figure 5, B and C) was apparently different from that of sorbitol particles (Figure 5A). The surface morphology of proliposomes at other SPC to sorbitol ratios was, on the other hand, rather similar to that of sorbitol particles. These results corroborated the excess SPC deposition on the surface of the first two formulations.

The flow properties of blank proliposomes with various amounts of SPC are shown in Table 3. The angle of repose is a characteristic related to interparticulate friction resulting in resistance to movement (Gustavo, 2005). The low angle of repose indicates free-flowing property of particles.

Table 3: Angles of repose and bulk densities of sorbitol and blank proliposome formulations. Data are shown as mean ± SD*.

Formulation	**SPC (mmol) / g of sorbitol**	**Angle of repose (degrees ± SD*)**	**Bulk density (g/cm^3)**
Sorbitol	-	34.09 ± 1.06	0.446
Blank proliposomes			
I	0.35	Not available	0.350
II	0.31	Not available	0.384
III	0.26	33.90 ± 1.77	0.383
IV	0.22	29.03 ± 1.87	0.472
V	0.18	24.36 ± 1.00	0.578

*From three measurements

Angles of repose of proliposomes containing 0.35 and 0.31 mmol of SPC/g of sorbitol were not available because the excess SPC on sorbitol particles completely hindered the flow of these particles. The transition temperature of SPC being -15°C (Cevc, 1993), the phospholipid is in the semisolid state at room temperature. The

excess SPC melted and proliposome particles stuck together, resulting in agglomeration. Because of the severe agglomeration of proliposome particles, the particles could not pass through the funnel.

The angles of repose of the other three proliposome preparations were similar to or less than that of sorbitol. Because of its hygroscopic property (Rowe et al., 2006), sorbitol tended to agglomerate, resulting in a moderate angle of repose. The angle of repose of sorbitol in this study was in the range classified as “good” flow property according to the United States Pharmacopeia (The United States Pharmacopeia Convention, 2012). When SPC was adsorbed onto the surface of sorbitol, its relatively hydrophobic nature prevented the moisture from coming into direct contact with the sorbitol particles. Thus, agglomeration of proliposomes with appropriate amounts of SPC was minimal.

The bulk density is a physical property related to particle size and packing. For hygroscopic substances, moisture sorption is generally associated with increased cohesiveness from inter-particle bridges, resulting in low bulk densities (Gustavo, 2005). Sorbitol particles are hygroscopic and, therefore, have a relatively low bulk density. In the case of proliposomes, the bulk density tended to increase as the percentage of SPC in the proliposome formulations decreased. The higher bulk density could indicate lower degree of particle agglomeration, and thus, the desired flowability.

The results from physical appearance, surface morphology, angle of repose, and bulk density all indicated that proliposomes containing 0.22 and 0.18 mmol/g of sorbitol were comparable or superior to sorbitol in terms of powder properties. However, from the physical appearance and flow properties, blank proliposomes containing 0.22 mmol of SPC/g of sorbitol still showed a tendency to agglomerate (Figure 4E and Table 3). Proliposomes containing 0.20 mmol of SPC/g of sorbitol were prepared. The physical appearance and electron micrograph of the formulation are shown in Figure 6.

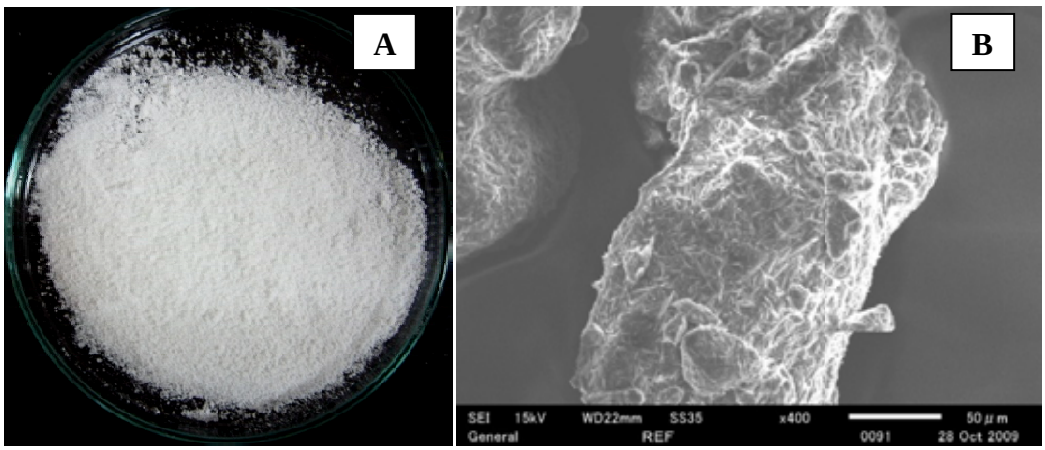


Figure 6: Physical appearance (A) and electron micrograph (B) of blank proliposomes containing 0.20 mmol of SPC/g of sorbitol

Blank proliposomes containing 0.20 mmol of SPC/g of sorbitol gave the angle of repose and the bulk density of 24.18 ± 1.20° and 0.501 g/cm^3, respectively. While the bulk density did not differ from the other two formulations above, the angle of repose showed a superior flow property. In this study, proliposomes with high bulk density, low angle of repose, and high SPC content would be desirable. Thus, the proliposome preparation containing 0.20 mmol of SPC/g of sorbitol was selected for further study.

Under the conditions used in this study, SPC loading was higher than that reported on proliposomes prepared with the slurry method, where SPC loading was around 0.14 mmol/g of sorbitol (Xu et al., 2009). A lower SPC loading of around 0.09 mmol/g of sorbitol was reported by Song et al. (2005), despite the use of the film-deposition-on-carriers method. The use of different carrier materials can also affect the SPC loading. When mannitol was used as the carriers, phospholipid loading could be as high as 0.5 mmol/g of mannitol (Yan-yu et al., 2006). Although mannitol could accommodate a higher amount of phospholipid, its water solubility is poorer than that of sorbitol (1 in 5.5 versus 1 in 0.5) (Rowe et al., 2006). Mannitol was not used in this study since higher water solubility was desired for the ease of proliposome hydration (Payne et al., 1986).

When the blank proliposomes containing 0.20 mmol of SPC/g of sorbitol came into contact with deionized water, liposomes were formed (Figure 7). Figure 7B shows liposome budding from proliposome particles. The cryogenic scanning electron micrograph of the hydrated proliposome preparation also confirmed that liposomes were properly formed from the proliposomes (Figure 7C).

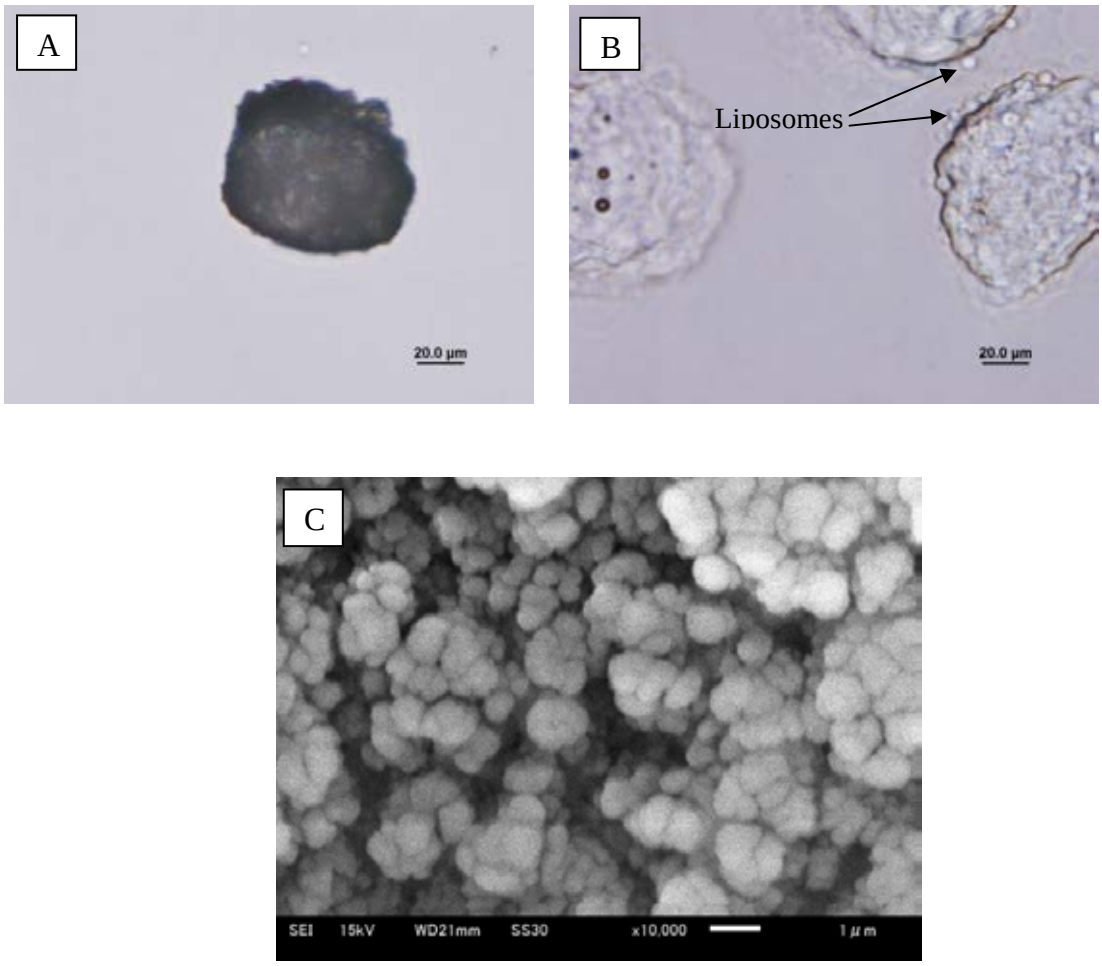


Figure 7: Formation of liposomes from proliposome particles of (A) a proliposome particle before hydration (×400), (B) liposome formation from the hydrated proliposome preparation (×400), and (C) cryogenic electron micrograph of liposomes formed after complete hydration of the proliposome preparation (×10,000)

1.2. Determination of SPC to CHO ratio

CHO is often required for the physical stability of liposomes entrapping hydrophilic substances (Wilschut, 1982). However, too much CHO may result in the disruption of bilayer formation (Szoka Jr. and Papahadjopoulos, 1980). Thus, blank proliposomes at various SPC:CHO ratios were prepared and the physical properties monitored. In addition, the feasibility of liposome formation upon contact with the aqueous phase was also assessed in order to determine the optimal ratios of the two lipids. The SPC:sorbitol ratio was 0.20 mmol/g as selected from the previous experiment. The physical appearance and electron micrographs of sorbitol and proliposomes containing SPC:CHO molar ratios of 1:0, 1:0.25, 1:0.5, and 1:1 are displayed in Figures 8 and 9, respectively.

Figure 8: Physical appearance of blank proliposomes with the SPC:CHO ratios of (A) 1:0, (B) 1:0.25, (C) 1:0.5, and (D) 1:1

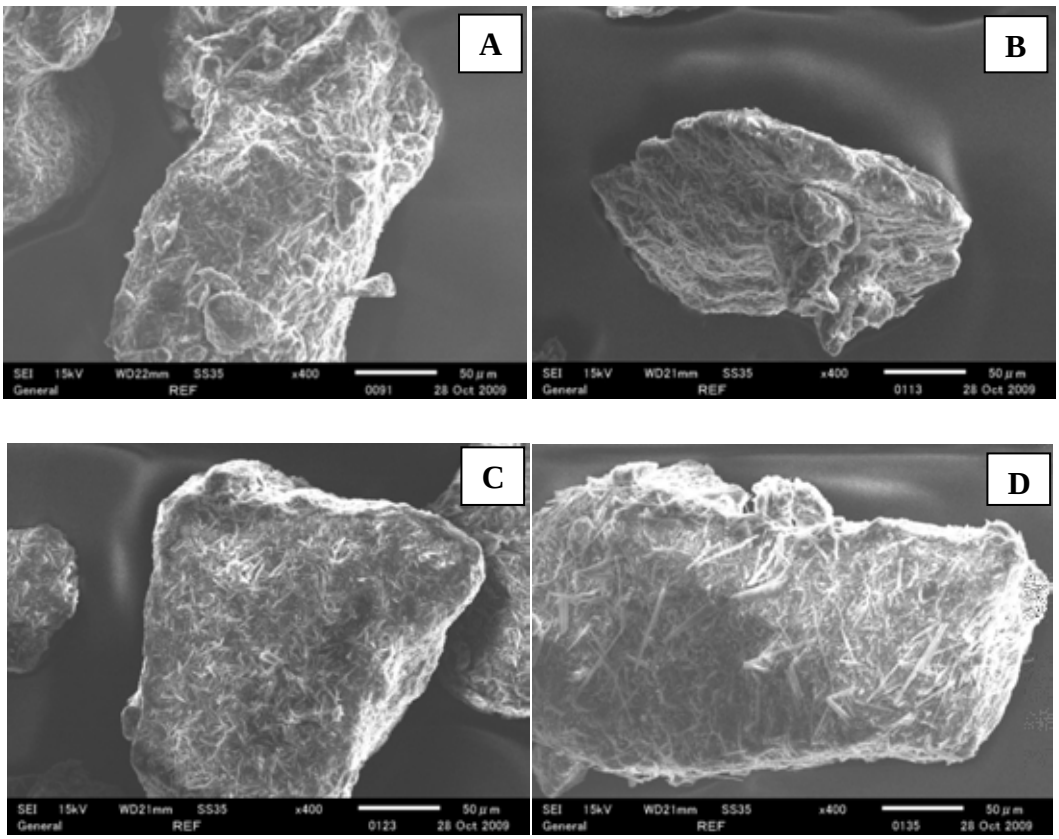


Figure 9: Electron micrographs of blank proliposomes with the SPC:CHO ratios of (A) 1:0, (B) 1:0.25, (C) 1:0.5, and (D) 1:1

The physical appearance and the electron micrographs of proliposomes with the SPC:CHO ratios of 1:0, 1:0.25, and 1:0.5 (Figure 9, A-C) were rather similar to those of sorbitol particles (Figures 4A and 5A). However, the physical appearance of proliposomes with SPC:CHO ratio of 1:1 showed some degree of agglomeration (Figure 8D). In addition, the electron micrograph of proliposomes with the SPC:CHO ratio of 1:1 was rather different from that of sorbitol particles. Needle-like crystals were clearly evident on the surface of proliposomes (Figure 9D). These structures suggested the deposition of excess CHO on the surface of proliposomes.

The flow properties of proliposomes with various SPC:CHO ratios are presented in Table 4.

Table 4: Angles of repose and bulk densities of sorbitol and blank proliposomes containing various ratios of SPC:CHO. Data are shown as mean ± SD*.

SPC:CHO	Angle of repose (degrees±SD*)	Bulk density (g/cm^3)
1:0	24.18 ± 1.20	0.501
1:0.25	24.39 ± 0.06	0.502
1:0.5	27.35 ±1.18	0.506
1:1	31.61 ±1.09	0.501
Sorbitol	34.09 ± 1.06	0.446

*From three measurements

The angles of repose of proliposomes in the first three formulations were less than that of sorbitol particles. When the CHO content reached 50 mol%, the angle of repose was much higher. The higher amount of CHO had a tendency to give the higher angle of repose, thus the poorer flow property. CHO melts at around 147-150°C (Rowe et al., 2006). At room temperature, CHO is in the solid form, and thus should not cause the agglomeration of proliposomes. However, the higher angle of repose implies the interparticulate friction or resistance to movement between particles (The United States Pharmacopeia Convention, 2012). From the electron micrographs, the appearance of needle-like crystals deposited on the surface of proliposomes increased with the increasing CHO contents. The needle-like structure was clearly seen at 50 mol% of CHO content. Thus, it was possible that these needle-like crystals could cause the interparticulate friction that resulted in the increased angle of repose.

Formation of liposomes from proliposomes is a crucial step for the success of proliposome formulations. Upon hydration of those formulations with Ultrapure® water at 37°C, the liposome dispersions were obtained. Figure 10 shows the liposome dispersions from blank proliposome formulations with the SPC:CHO ratios of 1:0, 1:0.25, 1:0.5, and 1:1 (from the left to right), respectively.

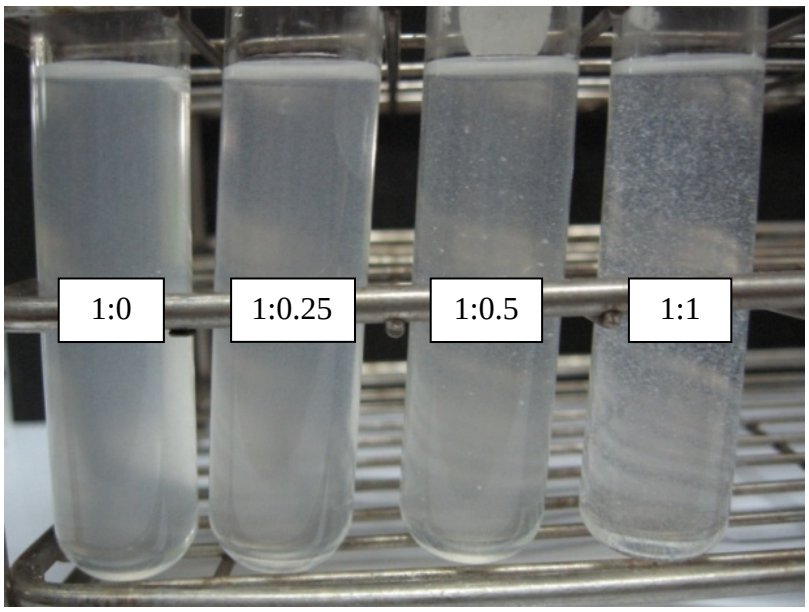


Figure 10: The resultant liposome dispersions from various proliposome preparations with the SPC:CHO ratios of 1:0, 1:0.25, 1:0.5, and 1:1, respectively

Figure 10 shows that the formulations with the SPC:CHO ratios of 1:0.5 and 1:1 could not be completely hydrated after 60 minutes at 37°C. White particles were evident in the resultant liposome dispersions. The appearance of these particles under a light microscope was consistent with the remnants of the lipid domain with an excess in CHO (Figure 11).

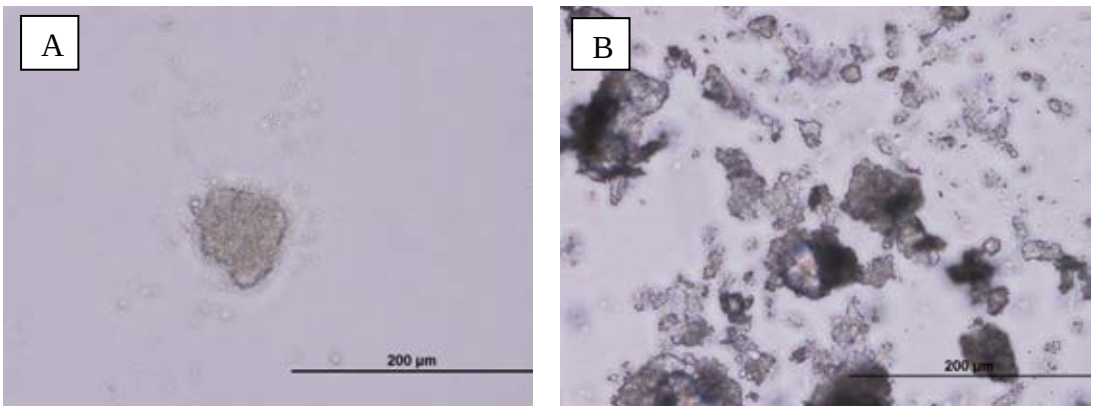


Figure 11: Photographs of (A) a representation of the white particles seen in the resultant liposome dispersions and (B) partially hydrated CHO particles (×200)

From the physical properties and the ease of liposome formation upon hydration, only proliposomes containing SPC:CHO at the molar ratios of 1:0 and 1:0.25 were further used in this study.

1.3. Characterization of P-gp substrate-loaded proliposomes

To assure that the P-gp substrate-loaded proliposomes retained the required physical properties of the blank proliposomes, the bulk density and the angle of repose of both calcein AM and acyclovir-loaded proliposomes were determined. The results are shown in Table 5.

Table 5: Angles of repose and bulk densities of P-gp substrate-loaded proliposomes. Data are shown as mean ± SD*.

P-gp substrate	SPC:CHO	Angle of repose (degrees ± SD*)	Bulk density (g/cm^3)
Calcein AM	1:0	25.56 ± 0.00	0.521
	1:0.25	22.61 ± 1.40	0.532
Acyclovir	1:0	25.78 ± 1.35	0.462
	1:0.25	25.01 ± 1.35	0.504

*From three measurements

Calcein AM-loaded proliposomes without CHO showed a slightly higher angle of respose than that of the corresponding blank proliposomes. On the other hand, the calcein AM-loaded proliposomes at 1:0.25 SPC:CHO ratio displayed a lower angle of repose than the corresponding blank proliposomes. Both formulations of calcein AM-loaded proliposome particles could be classified as “excellent” with respect to the flow property based on the angle of repose (see Appendix I) (The United States Pharmacopeia Convention, 2012). The bulk densities of both calcein AM-loaded proliposomes were also better than those of the corresponding blank proliposome formulations.

For acyclovir-loaded proliposomes, the angles of repose of both formulations were slightly higher than that of the corresponding blank formulations. The bulk density of acyclovir-loaded proliposomes without CHO was lower than that of the

corresponding blank proliposome formulation. The bulk density of acyclovir-loaded proliposomes at the 1:0.25 ratio of SPC:CHO were comparable to that of the corresponding blank proliposomes.

The angles of repose and the bulk densities of all 4 formulations of calcein AM- and acyclovir-loaded proliposomes indicated the better flow properties of these formulations when compared to sorbitol. Thus, all these P-gp substrates-loaded proliposome formulations were used for further experiments.

1.4 Stability of acyclovir-loaded proliposomes

The purpose of this experiment was to investigate how long acyclovir-loaded proliposomes could be kept under the storage condition before being used in the uptake study. Acyclovir-loaded proliposomes with the SPC:CHO ratios of 1:0 and 1:0.25 were prepared and evaluated for physical stability with respect to acyclovir entrapment efficiency of the resultant liposome dispersions (Yan-yu et al., 2006; Xu et al., 2009). A preliminary study indicated that the entrapment efficiency decreased more than 20% within a month. Thus the experiment was designed to evaluate the physical stability of acyclovir-loaded proliposomes over a period of two weeks. The results from three batches of each proliposome formulation are displayed in Figure 12.

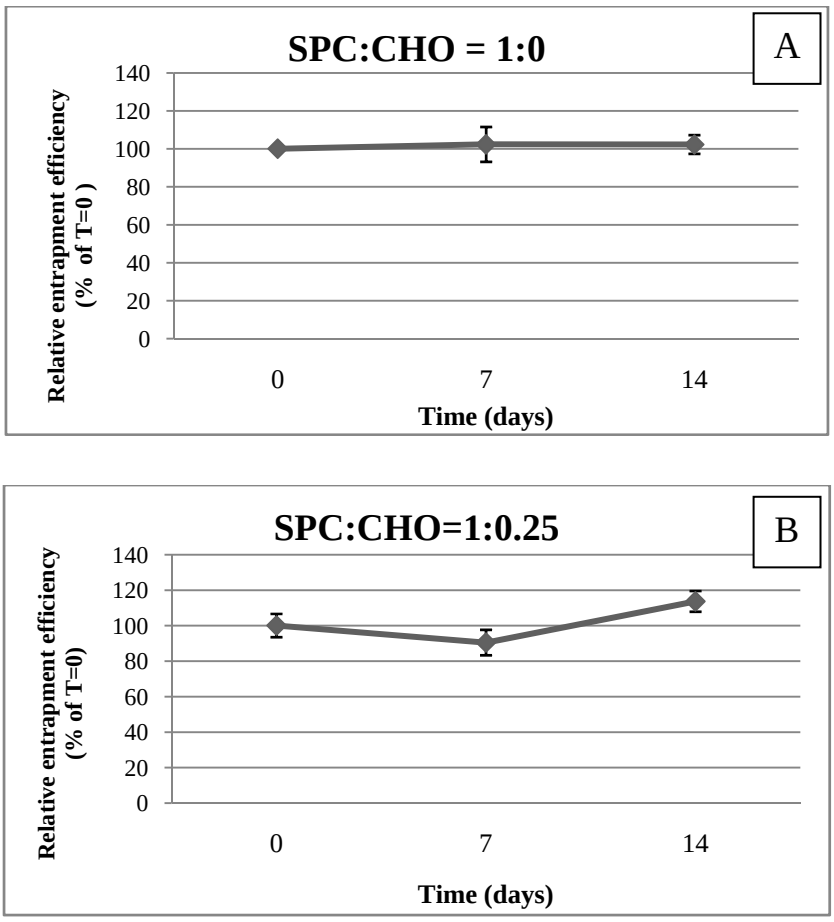


Figure 12: Entrapment efficiency data of hydrated acyclovir-loaded proliposomes at the SPC:CHO ratios of (A) 1:0 and (B) 1:0.25 at 0, 7, and 14 days of storage (n = 3, the data are presented as mean ± SEM)

From the data, there was no statistically significant difference in the entrapment efficiency within 14 days of storage for both formulations. While an earlier work notes that proliposome preparation can be stable at room temperature for 6 months (Xu et al., 2009), it was not the case here. Acyclovir-loaded proliposomes could be kept under N_2 at -20°C, protected from light, for less than one month. It was possible that the method used for proliposome preparation had a profound effect on the stability of proliposomes. Vinpocetine-loaded proliposomes, prepared by the slurry method, were reported to be stable at room temperature for 6 months (Xu et al., 2009). The total time for preparation of vinpocetine-loaded proliposomes was only around 20 minutes, while the acyclovir-loaded proliposomes prepared by film deposition on carriers in our laboratory took around 2 hours. The longer preparation

process could expose the phospholipid to the unfavorable environment (relatively high temperature with some light exposure) that was detrimental to SPC integrity. Thus, the stability of acyclovir-loaded proliposomes was compromised. As a result, all acyclovir-loaded proliposome preparations were used within a few days after preparation.

2. Effect of the mSIF medium on P-gp function

In this study, proliposomes were developed to use as delivery systems for the oral route. Thus, the simulated intestinal fluid, TS (SIF) (The United States Pharmacopeia Convention, 2012) was first selected to be used as the hydrating medium and the uptake medium. Unfortunately, the result from the preliminary experiment showed that the use of the SIF as the uptake medium resulted in the detachment of the cells from the culture plates. Therefore, a modified medium was used instead. The monobasic potassium phosphate, the main composition of the SIF was replaced with the biological buffer HEPES at an equal concentration (Perrin and Dempsey, 1974). However, HEPES can also have an effect on P-gp function by increasing the ATPase activity (Luo et al., 2010). The P-gp function was thus evaluated when the mSIF was used as the uptake medium. The serum-free DMEM was used as the control medium.

The accumulation of the fluorescent P-gp substrate rhodamine 123 in Caco-2 cells with and without verapamil was monitored and expressed as the reverse ratio. The reverse ratio was defined here as the ratio between the concentrations of the P-gp substrate accumulated in the cells in the presence and the absence of the P-gp inhibitor. The results are shown in Table 6.

Table 6: Intracellular accumulation of rhodamine 123 in Caco-2 cells. Data are shown as mean ± SD.

Uptake medium	Intracellular accumulation of rhodamine 123 (ng/ml)		Reverse ratio
	Without verapamil	With verapamil	
DMEM	35.41 ± 3.01	82.57 ± 4.99	2.33
mSIF	93.90 ± 5.57	131.44 ± 6.59	1.40

The reverse ratio with the serum-free DMEM was 1.66 fold of that with mSIF, indicating that the components in the mSIF could obscure the function of P-gp. Besides, the osmolality of the mSIF was only 89 mosmol per kg, which was well below the recommended values (260-320 mosmol per kg) for cell culture in general practice (Freshney, 2005). Thus, the serum-free DMEM was mixed at various concentrations with the mSIF and further evaluated to be selected as the uptake medium.

3. Selection of the uptake medium

The reverse ratios of rhodamine 123 uptake in the mixtures of mSIF and DMEM at various ratios are shown in Table 7.

Table 7: Reverse ratios of rhodamine 123 uptake from various compositions of the uptake medium

mSIF:DMEM	Intracellular accumulation of rhodamine 123 (ng/ml)		Reverse ratio	Osmolality (mosmol/kg)
	without verapamil	with verapamil		
0:100	34.65 ± 0.40	72.06 ± 1.16	2.08	311
25:75	37.04 ± 0.33	67.69 ± 1.95	1.83	277
50:50	35.47 ± 0.29	68.16 ± 1.78	1.92	210
75:25	38.28 ± 0.49	66.60 ± 1.60	1.74	152

The reverse ratio of rhodamine 123 uptake into the cells of 1.5 has been accepted as an indication of P-gp function (Zastre et al., 2002). The data in Table 7 indicated that the mSIF could be used in the uptake medium at concentrations up to 75% since the reverse ratios were still greater than 1.5. However, at 75% mSIF, the osmolality of the medium was still much lower than the recommended values. The osmolality at 50% mSIF was still lower than that recommended for the cultivation of animal cells. However, at higher concentration of the serum-free DMEM, the hydration of proliposomes was extremely difficult. In addition, all the uptake experiments with Caco-2 cells in this study were carried out within a relative short period of time. The incubation time used for each experiment was 2.5 hours at most. Thus, the composition of the uptake medium consisting of 50% mSIF in serum-free DMEM was selected as the uptake medium in all uptake experiments.

4. Verification of Caco-2 cells

4.1. Verification of P-gp function

The uptake of calcein AM and rhodamine 123 has been commonly used to evaluate the function of P-gp (Utoguchi et al., 2000; Zastre et al., 2002). Verification of the P-gp function was routinely monitored in every uptake experiment. The means and SD of the reverse ratios from the uptake experiments are shown in Table 8.

Table 8: Reverse ratios of calcein AM and rhodamine 123 with different P-gp inhibitors

P-gp substrate	P-gp inhibitor	Reverse ratio (mean ± SD)*
calcein AM	verapamil	1.52 ± 0.24
	cyclosporine A	2.26 ± 0.34
rhodamine 123	verapamil	1.60 ± 0.17
	cyclosporine A	1.71 ± 0.10

* The data were from 3-6 experiments.

The reverse ratios from the uptake experiments were close to or higher than 1.5 in all experiments with both P-gp substrates. These numbers are in consistent with the previous reports on P-gp function with uptake studies (Eneroth et al., 2001; Zastre

et al., 2002; Ing-orn Prasanchaimontri, 2009). Inhibition with cyclosporine A tended to give higher reverse ratios when compared to those inhibited with verapamil. Thus, under the present culture condition, Caco-2 cells adequately exhibited the required P-gp function.

4.2. Verification of enhanced accumulation of fluorescent markers in Caco-2 cells via liposome delivery

4.2.1. Effect of liposome concentration on the accumulation of calcein in Caco-2 cells

To verify that Caco-2 cells used in this study were capable of liposome uptake, calcein was used as the aqueous marker for liposomes. Figure 13 shows the accumulation of calcein in Caco-2 cells from calcein solution and calcein-loaded liposomes at various concentrations.

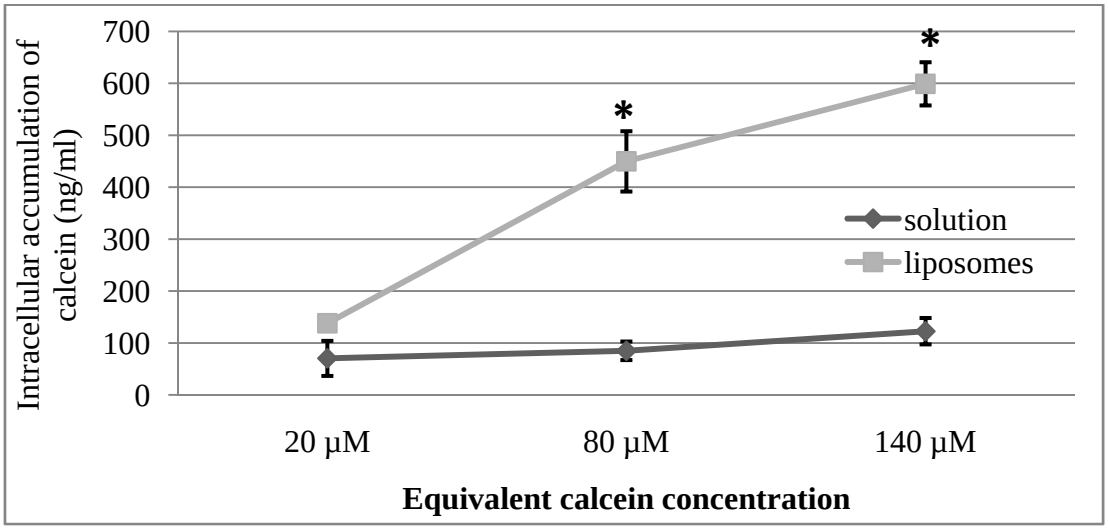


Figure 13: Intracellular accumulation of calcein from calcein solution and calcein-loaded liposomes at various liposome concentrations, expressed as the equivalent calcein concentration. The lipid concentrations were 0.0125-0.085 mg/ml. Data are shown as mean ± SEM.

The expected mechanism of liposome uptake by living cells is usually by endocytosis, which is a saturable process (Straubinger et al., 1983; Sahay, Alakhova, and Kabanov, 2010). In this present study, the profile of calcein uptake tapered off with increasing concentrations of calcein-loaded liposomes. On the contrary, the

calcein solution gave a linear calcein uptake pattern with increasing calcein concentrations. The calcein accumulation in Caco-2 cells was higher from the liposomes than from the solution at all corresponding concentrations. The statistically significant differences were seen at the equivalent calcein concentrations of 80 and 140 μM ($p < 0.05$). These results were consistent with endocytosis as the uptake mechanism. It is worth noting that much higher uptake values were seen with liposomes despite the size heterogeneity of the preparation. These results indicated that Caco-2 cells used in this study were capable of taking up liposomes by a pattern consistent with endocytosis. This process was more efficient than the simple diffusion of the fluorescent calcein marker.

4.2.2. Effect of size distribution profile on the accumulation of calcein AM in Caco-2 cells

In this study, blank proliposomes gave liposomes with size heterogeneity, ranging from less than 100 nm to around 10 μm (see Appendix J). The aim of this experiment was to investigate the ability of the Caco-2 cells to uptake liposomes with the difference in size distribution profiles since the hydration of proliposomes would result in the heterogeneity in the size of liposome vesicles. In addition, most of the studies evaluating cellular uptake of liposomes were usually done using liposomes with controlled sizes (Anderson et al., 2000; Goren et al., 2000; Li et al., 2011; Song et al., 2011). The uptake of calcein AM from solution, liposomes prepared by the film hydration method, and liposomes extruded through 100-nm polycarbonate membranes was compared (Figure 14).

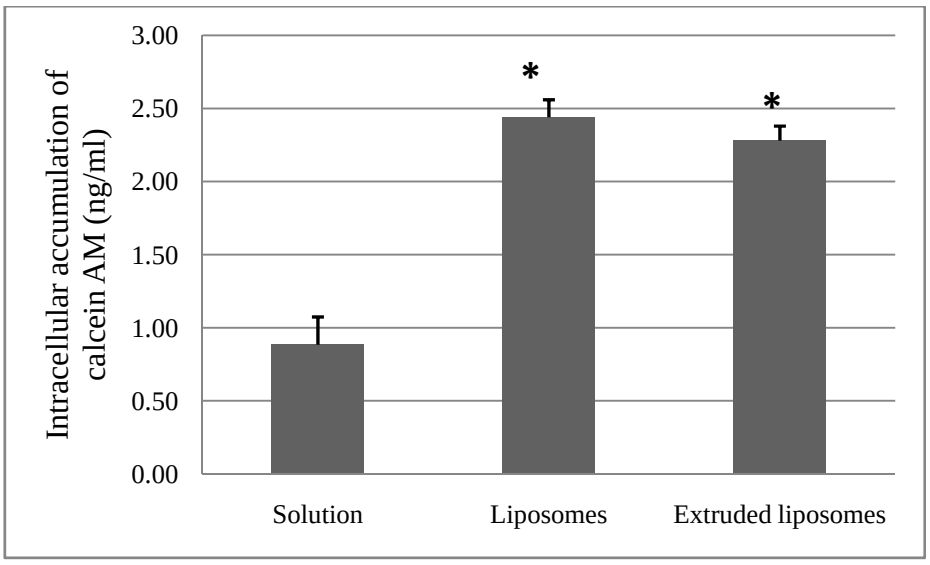


Figure 14: Intracellular accumulation of 25 nM calcein AM from solution, calcein AM-loaded liposomes, and calcein AM-loaded extruded liposomes. The lipid concentration was 0.35 mg/ml in both liposome formulations. Data are shown as mean ± SEM (n = 3).
* $p < 0.05$, compared with calcein AM solution

The intracellular accumulation of calcein AM from the liposomes and the extruded liposomes was significantly higher than that of the solution ($p < 0.05$). However, there was no statistical difference between the uptake of calcein AM from the calcein AM-loaded liposomes and the extruded calcein AM-loaded liposomes. From the preliminary study, the particle size of the extruded liposomes was around 120 nm (see Appendix J). On the other hand, the particle size of the unextruded liposomes prepared from the film hydration method is known to be heterogeneous. The size range of unextruded liposomes prepared by this method can vary from nanometers to several micrometers (Mui and Hope, 2007). However, in a previous study with liposomes in bone marrow macrophages, the increase in liposome size resulted in the decrease in the uptake of liposomes (Allen et al., 1991). The discrepancy could be attributed partly to the difference in the uptake mechanisms (endocytosis versus phagocytosis) between the two types of the cells (Caco-2 cells versus macrophages). In this present study, the difference in size distribution profiles did not affect the uptake of liposomes by Caco-2 cells under the conditions used. In

addition, both liposome systems could efficiently enhance the intracellular accumulation of calcein AM when compared with the solution. The results indicated that it was plausible for Caco-2 cells to take up liposomes with size heterogeneity, which were expected to form after the hydration of proliposome preparations.

5. Effects of proliposome systems on the accumulation of P-gp substrates in Caco-2 cells

5.1. Uptake of calcein AM from proliposome systems

In this study, it was hypothesized that proliposome systems could enhance the intracellular accumulation of the hydrophobic P-gp substrate calcein AM. The result from the previous experiment showed that the liposome systems could enhance the intracellular accumulation of calcein AM regardless of size distribution. Reports with respect to the role of liposomes in overcoming the function of the efflux transporter P-gp are numerous (Huwyler et al., 2002; Sadava, Coleman, and Kane, 2002; Mamot et al., 2003). However, there was no study that directly assessed this overcoming effect of proliposome delivery systems. Most studies with proliposomes were directed to both hydrophobic and hydrophilic drugs that are not P-gp substrates such as salmon calcitonin (Song et al., 2005), silymarin (Yan-Yu et al., 2006), cromolyn sodium (Deshmukh et al., 2008), and vinpocetine (Xu et al., 2009). In this present study, the extents of calcein AM uptake from the extruded calcein AM-loaded liposomes and the calcein AM-loaded proliposomes were compared. The calcein AM solution was used as the control. The results of the liposome and proliposome preparations without CHO are shown in Figure 15.

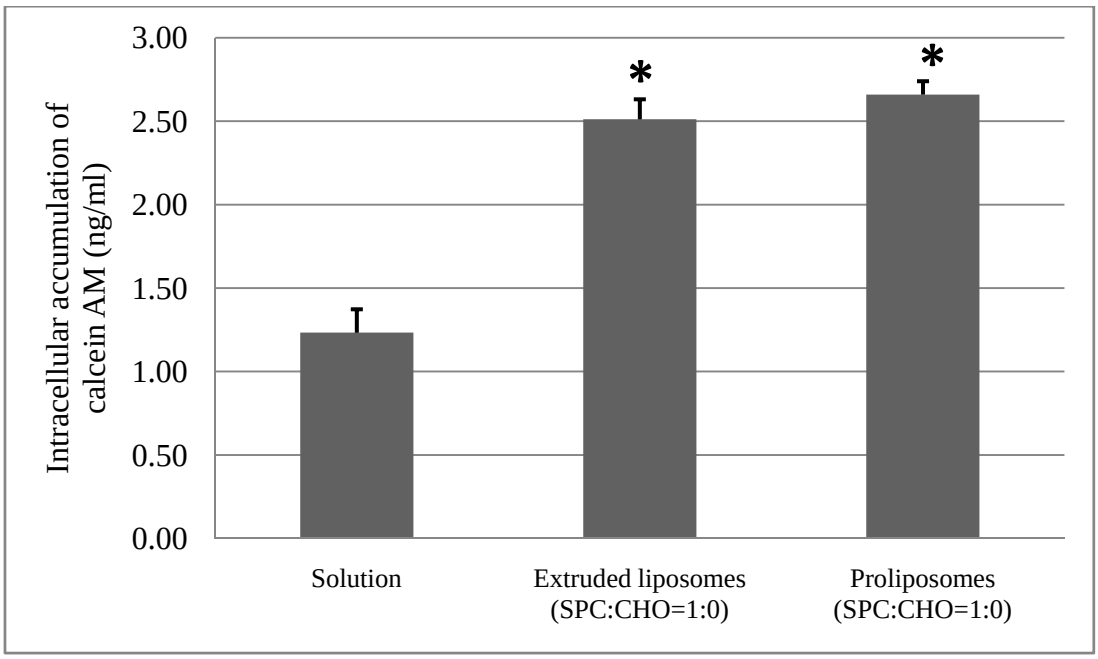


Figure 15: Intracellular accumulation of 25 nM calcein AM from solution, calcein AM-loaded liposomes (extruded), and calcein AM-loaded proliposomes. The liposome and proliposome preparations were those without CHO (total lipid = 0.35 mg/ml). Data are shown as mean ± SEM (n = 6).
*$p < 0.05$, compared with the calcein AM solution

The intracellular accumulation of calcein AM from both the extruded liposomes and hydrated proliposomes was significantly higher than that of the solution ($p < 0.05$). However, there was no statistical difference between the uptake from the extruded liposomes and the proliposomes. These results indicated that the proliposome preparation was at least as efficient as the extruded liposomes in delivering the hydrophobic P-gp substrate into Caco-2 cells. When the ease of preparation and handling was considered, the proliposome system would be more practical for further development. Though the stability of proliposomes would still be a problem, it would also be possible to improve the stability by selecting a more favorable method of preparation. Some other methods of proliposome preparation with much better product stability have been reported (Xu et al., 2009).

Figure 16 displays the intracellular accumulation of calcein AM from the calcein AM solution and the resultant liposomes formed from the calcein AM-loaded proliposome preparations containing either no CHO or 20 mol% of CHO. The calcein

AM accumulation from the resultant liposomes with and without CHO was significantly higher than that of the solution ($p < 0.05$). In addition, the calcein AM accumulation from proliposomes with 20 mol% of CHO was also significantly higher than that from the proliposomes without CHO. Proliposomes could enhance the accumulations of calcein AM in Caco-2 cells for 2.16 and 2.55 fold for the preparation without CHO and with 20 mol% of CHO, respectively.

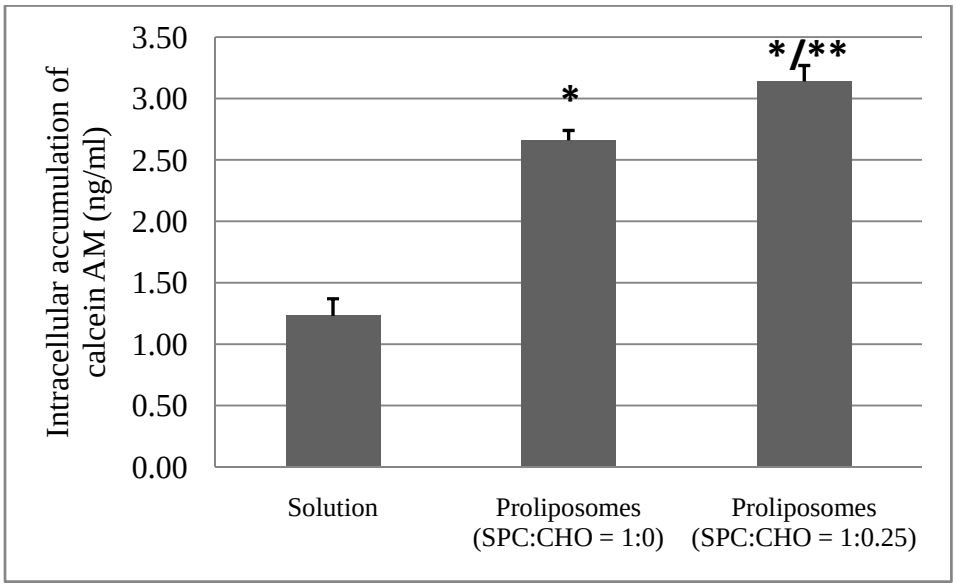


Figure 16: Intracellular accumulation of 25 nM calcein AM from the solution and the calcein AM-loaded proliposome preparations without CHO and with 20 mol% of CHO (total lipid = 0.35 mg/ml). Data are shown as mean ± SEM (n = 6).

*$p < 0.05$, compared with calcein AM solution

**$p < 0.05$, compared with calcein AM-loaded proliposomes without CHO (SPC:CHO 1:0)

Proliposome delivery systems could enhance the absorption of hydrophobic molecules including silymarin (Yan-yu et al., 2009), exemestane (Hiremath et al., 2009), and vinpocetine (Xu et al., 2009). This present finding is consistent with these previous studies. The mechanism by which proliposomes increase the absorption of these hydrophobic drugs is proposed to be the vesicle transport via endocytosis along with the increase in drug dissolution (Hiremath et al., 2009; Janga et al., 2011; Xu et

al., 2009; Yan-yu et al., 2009). Increased lymphatic transport is also speculated (Xu et al, 2009).

The calcein AM-loaded proliposomes containing CHO could better enhance the intracellular accumulation of calcein in Caco-2 cells. Incorporation of CHO into the bilayer of liposomes affects the membrane fluidity. Above the transition temperature of phosphatidylcholine (around -15°C in this case), CHO incorporated into the membrane of liposomes results in condensing of the phospholipid bilayer and decreasing the membrane fluidity (New, 1989). In a previous study, the incorporation of CHO into liposomal bilayers resulted in retention of the P-gp substrate rhodamine 123 within the cells better than the liposome preparation without CHO (Kang et al., 2009). The authors reasoned that the enhanced liposome membrane integrity retarded the release of rhodamine 123 from the vesicles, thus preventing the substrate to interact with P-gp. However, this scenario would be applied only to rhodamine 123, which is a relatively hydrophilic P-gp substrate. An alternative explanation would be needed for more hydrophobic compounds such as calcein AM. In a previous study, CHO was found to increase P-gp-mediated ATP hydrolysis, resulting in inhibition of the function of the transporter (Wang et al., 2000). This might explain in part the better result seen with the proliposomes containing 20 mol% of CHO.

5.2. Uptake of acyclovir from proliposome systems

Several studies have indicated that acyclovir is a substrate of P-gp (De Vrueh et al., 1998; Salama et al, 2004; Zhao-Gang et al., 2004; Palmberger et al., 2008). Acyclovir was selected as a model hydrophilic P-gp substrate in this present study because the drug is in the BCS class III (Lindenberg et al., 2004), where permeability usually limits drug absorption. The bioavailability of acyclovir is only 15-30% (Lacy et al., 2005). Thus, if proliposomes could circumvent P-gp and/or increase the uptake of the drug via vesicular transport, the effect would be more unambiguously seen. Acyclovir is not a substrate for the CYP 450 enzymes (U.S. FDA, 2012). The poor permeability from its hydrophilicity (Lindenberg et al., 2004) along with the drug efflux by P-gp (De Vrueh et al., 1998; Salama, et al., 2004; Palmberger et al.,, 2008) contributes largely to acyclovir low bioavailability.

When the concentration of proliposomes was varied such that acyclovir concentration range from 0.54-1.05 mM, the uptake of acyclovir increased as the concentration increased up to 0.88 mM (Figure 17). However, when the uptake from acyclovir proliposomes was compared with that from the solution at the corresponding concentration acyclovir of 1.0 mM, no difference in the uptake of these preparations was seen, regardless of the proliposome CHO content (Figure 18). This was also the case with the extruded acyclovir-loaded liposomes (Figure 19). These results indicated that proliposomes, as well as liposomes, could neither circumvent the P-gp efflux nor increase the uptake of acyclovir via vesicular transport at the concentration used.

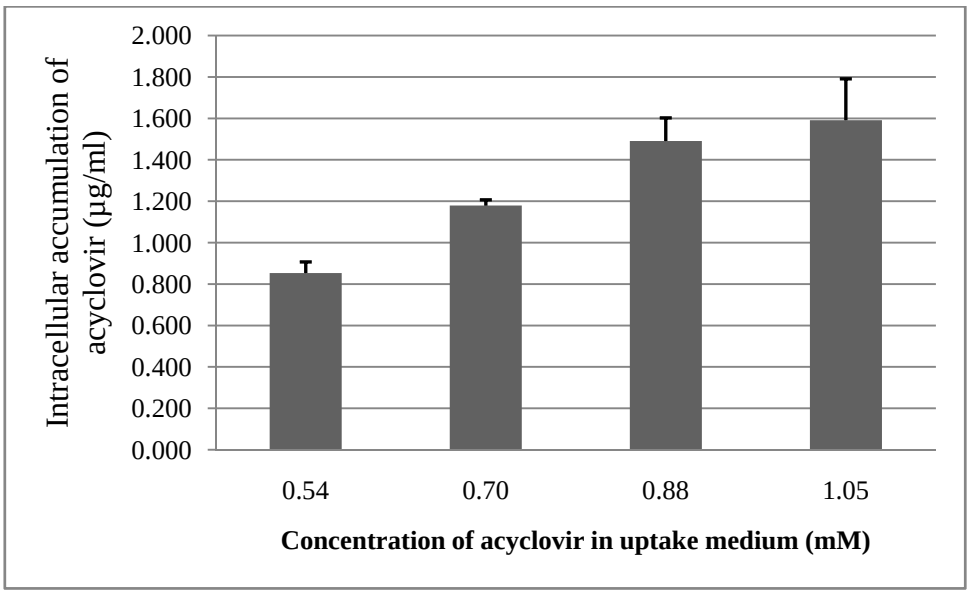


Figure 17: Intracellular accumulation of acyclovir from acyclovir-loaded proliposomes at various proliposome concentrations corresponding to SPC concentrations of 0.19, 0.24, 0.31, and 0.37 mg/ml, respectively. Data are shown as mean ± SD.

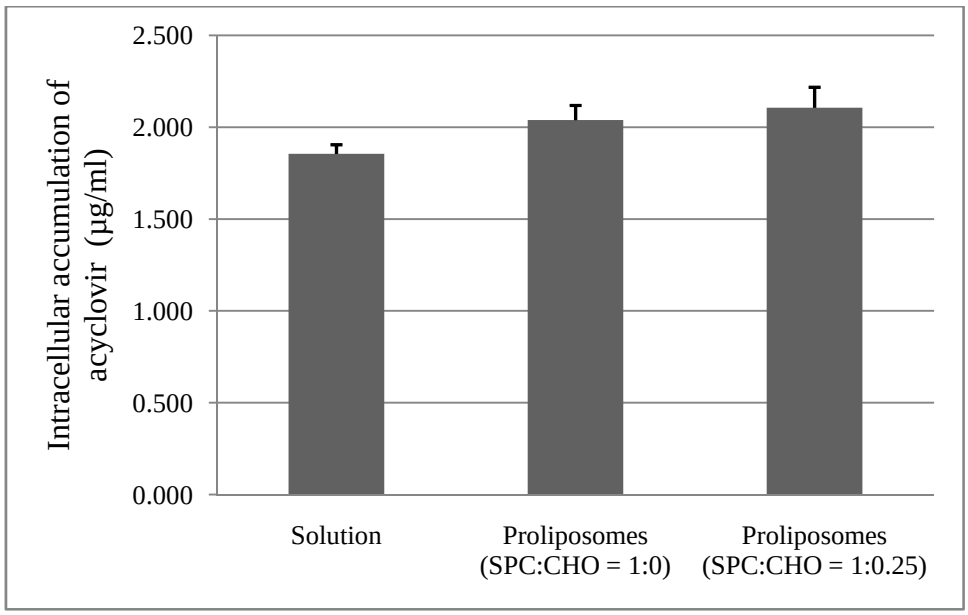


Figure 18: Intracellular accumulation of acyclovir (1 mM) from solution and the proliposomes with the SPC:CHO ratios of 1:0 and 1:0.25, respectively. The lipid concentration used in the experiment was 0.35 mg/ml. Data are shown as mean ± SEM (n = 3).

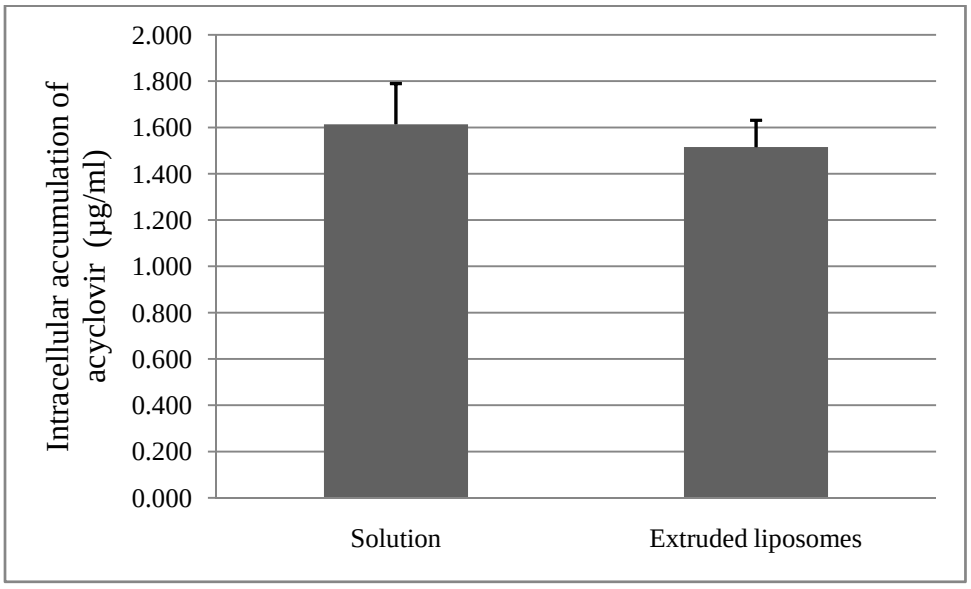


Figure 19: Intracellular accumulation of 1 mM acyclovir from the solution and the extruded liposomes (0.35 mg of SPC/ml). Data are shown as mean ± SEM.

The results seen with acyclovir could be explained partly by the low entrapment efficiency (EE) of the resultant liposomes formed from proliposomes after hydration. The EE values of the resultant acyclovir liposomes with and without CHO were 3.71 ± 0.42 and 3.84 ± 0.07% at the total lipid concentration 0.35 mg/ml, respectively. The increased uptake of hydrophilic compounds via endocytosis was evident with calcein liposomes despite the even lower lipid concentrations (see Figure 13). The most probable explanation for the enhancement in calcein uptake seen with liposomes would be the high entrapment of calcein in the liposome preparation used in this study. The calcein liposomes were hydrated with a rather concentrated calcein solution (80 mM). Upon dilution by almost a thousand fold for the uptake study, most calcein in the liposome preparation would be that entrapped in the vesicles. Thus, vesicle uptake via endocytosis resulted in the high extent of calcein in the cells. In addition, calcein is a highly hydrophilic compound. Log P of calcein is -5.02 (Gillet et al., 2011). The permeability by passive diffusion via the transcellular route is usually low for highly hydrophilic compound (Kerns and Di, 2008). The calcein uptake by passive diffusion through the cell membrane was inefficient in Caco-2 cells (see Figure 13). Thus, endocytosis of liposomes had a profound effect on calcein uptake. However, the high entrapment efficiency of hydrophilic compounds would be difficult to achieve with proliposomes. The process of in situ liposome formation would be a constraint on high drug entrapment since drug loading in proliposomes was also limited. More evidence where proliposomes were not beneficial for hydrophilic compounds was seen in a previous research on salmon calcitonin (Song et al., 2005). The authors found no increase in the delivery of salmon calcitonin from proliposomes without an absorption enhancer in rats.

To corroborate these results further, additional experiments were carried out. In every experiment, the presence of P-gp was verified using rhodamine 123 as the P-gp substrate. When cyclosporine A was used as the P-gp inhibitor, the average reverse ratio of rhodamine 123 was 1.70 ± 0.10 in the experiments with acyclovir. However, when acyclovir solution was tested in the presence of the P-gp inhibitor verapamil at 100 µM, the reverse ratio was close to 1 (Figure 20). A similar result was also seen when cyclosporine A was used (Figure 21).

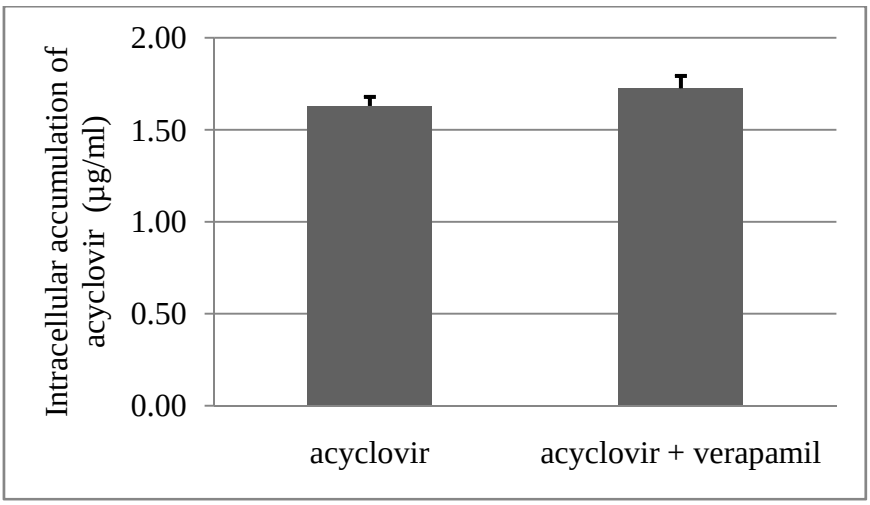


Figure 20: Intracellular accumulation of 1 mM acyclovir with and without 100 µM verapamil. Data are shown as mean ± SEM (n = 3).

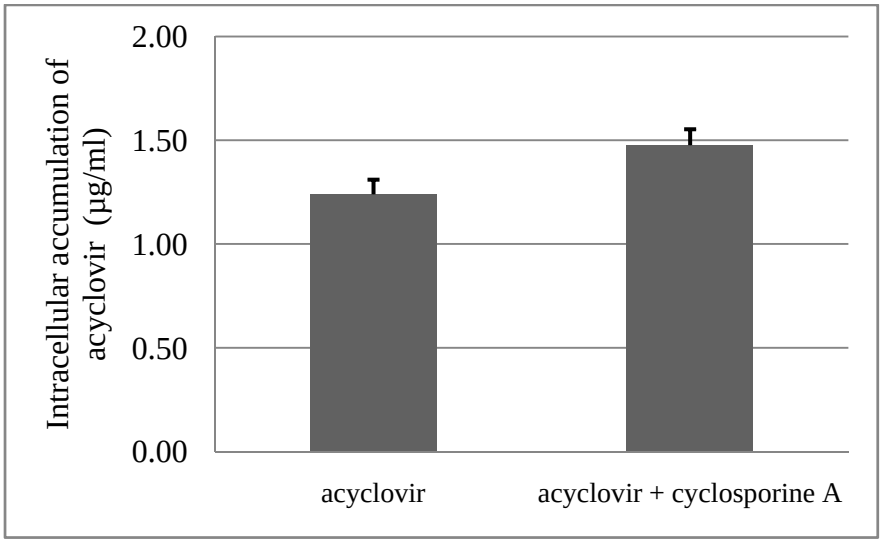


Figure 21: Intracellular accumulation of 1 mM acyclovir with and without 5 µM cyclosporine A. Data are shown as mean ± SD

The results with acyclovir solution in the presence of P-gp inhibitors suggested that the effect of P-gp efflux on acyclovir uptake was undermined despite its being a P-gp substrate (Salama et al., 2004; Palmberger et al., 2007).

Troutman and Thakker (2003) found that the addition of GW918, a P-gp inhibitor, had a profound effect on the secretory, but not the absorptive, permeability of the P-gp substrates rhodamine 123 and doxorubicin. The authors further proposed that these P-gp substrates were taken up into the cells via carrier-mediated transporters on the basolateral membrane. This scenario was contradictory to the results seen with acyclovir in this present study, though other influx transporters for acyclovir might also be involved. Acyclovir is taken up into cells by OCT1 (Takeda et al., 2002) and ENT proteins (Franco et al., 2008). Both proteins are expressed in Caco-2 cells (Hayeshi et al., 2008). Verapamil is a known inhibitor of OCT1 (Lee and Kim, 2004). Theoretically, it was possible that the effect of verapamil on the efflux transporter was counter-balanced by its effect on the influx transporters. However, further scientific evidence is needed to support this proposition. In addition, there is no evidence at the moment that cyclosporine A can inhibit any of these influx transporters. Thus, some other unknown mechanisms must have been responsible.

CHAPTER V

CONCLUSIONS

In this study, the feasibility of proliposomes as delivery systems for P-gp substrates via the oral route was accessed. The effects of proliposome compositions on physical properties of proliposomes and the resultant liposomes formed after hydration of the proliposomes were evaluated. Calcein AM and acyclovir were used as the model hydrophobic and hydrophilic P-gp substrates, respectively. The use of proliposome delivery systems to improve the accumulation of the model P-gp substrates in Caco-2 cells was also investigated.

Proliposomes were prepared by film deposition on carriers. In order to prepare proliposomes with acceptable flow properties, the ratio of SPC to the carrier sorbitol was titrated. The maximum SPC that could be loaded onto one gram of the sorbitol particles was 0.20 mmol for blank proliposomes. At this ratio, the surface morphology of the proliposomes was similar to that of the sorbitol particles. The flow properties with respect to the angle of repose and the bulk density were better than those of the carrier. Upon contact with the aqueous phase, liposomes rapidly formed from the proliposomes. CHO was conveniently included in the proliposome formulation at 20 mol% without alteration in the ease and completeness of the hydration process.

Inclusion of the model P-gp substrates did not impose any apparent adverse effects on the proliposomes. All formulations of P-gp substrate-loaded proliposomes still displayed better flow properties than those of sorbitol. However, the results of this present study indicated that the stability of these proliposome formulations could be problematic despite their storage under relatively stringent conditions.

To evaluate the role of proliposomes in promoting the absorption of P-gp substrates, the uptake studies were carried out in Caco-2 cells. The cells were verified to manifest sufficient P-gp function as well as the ability to take up efficiently the vesicles with heterogeneity in size. The uptake of calcein AM into Caco-2 cells from calcein AM-loaded proliposomes was more efficient that the uptake from solution. The degree of calcein AM uptake from proliposomes was comparable to that from the extruded liposomes. Thus, proliposomes seemed to be a feasible means of delivering hydrophobic P-gp substrates to Caco-2 cells. On the other hand, no advantage of

proliposomes over the solution was seen with the hydrophilic P-gp substrate acyclovir. This result could be only partly explained by the low entrapment of the drug. It was worth noting that, despite the verification of P-gp function in every experiment, verapamil and cyclosporine A failed to increase the accumulation of acyclovir when the drug was exposed to the cells as a solution. It was likely that other transporters might be involved in acyclovir uptake, which could confound the effect of the P-gp inhibitors. This finding still needs further clarification beyond the aims of this present study.

In conclusion, the overall results of this study indicated that it was plausible to use proliposomes to increase the cellular uptake of hydrophobic P-gp substrates. On the contrary, the advantage of proliposomes for hydrophilic P-gp substrates was not as convincing. To develop successful proliposome systems for hydrophilic compounds, it would be necessary to increase drug entrapment in the resultant liposomes after the hydration of proliposomes. This could be done by a number of strategies such as by means of surface charge interaction. However, since P-gp substrates vary widely in their structures, it would not be possible to design a universal proliposome system. Thus, further formulation development should be carefully designed to accommodate each of P-gp substrate of interest.

In order to further develop proliposomes for drug delivery via the oral route, the problem with stability has to be addressed. Shortening the processing time would be one of the strategies to improve proliposome stability. Other shortcomings, for example degradation by the stomach acid, could be overcome by the use of pharmaceutical technology such as the enteric coating.

REFERENCES

Ahn, B. -N., Kim, S. -K., and Shim, C.-K. 1995. Proliposomes as an intranasal dosage form for the sustained delivery of propranolol. Journal of Controlled Release, 34(3): 203-210.

Alam, M. A., Al-Jenoobi, F. I., and Al-Mohizea, A. M. 2012. Everted gut sac model as a tool in pharmaceutical research: limitations and applications. Journal of Pharmacy and Pharmacology, 64(3)**:** 326-336.

Allen, T. M., Austin, G. A., Chonn, A., Lin, L., and Lee, K. C. 1991. Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1061(1)**:** 56-64.

Anderson, K. E., Eliot, L. A., Stevenson, B. R., and Rogers, J. A. 2000. Formulation and evaluation of a folic acid receptor-targeted oral vancomycin liposomal dosage form. Pharmaceutical Research, 18(3)**:** 316-322.

Annakula, D., Errabelli, M. R., Jukanti, R., Bandari, S., and Veerareddy, P. 2010. Provesicular drug delivery systems: An overview and appraisal. Archives of Applied Science Research, 2(4)**:** 135-146.

Araya Lukanawonakul. 2005. Evaluation of liposomal composition on delivery of hydrophilic substances and P-glycoprotein substrates. Master's Thesis Department of Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Ariën, A., Goigoux, C., Baquey, C., and Dupuy, B. 1993. Study of in vitro and in vivo stability of liposomes loaded with calcitonin or indium in the gastrointestinal tract. Life Sciences, 53(16): 1279-1290.

Bailey, C. A., Bryla, P., and Malick, A. W. 1996. The use of intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. Advanced Drug Delivery Reviews, 22: 85-103.

Bansal, T., Akhtar, N., Jaggi, M., Khar, R. K., and Talegaonkar, S. 2009a. Novel formulation approaches for optimising delivery of anticancer drugs based on P-glycoprotein modulation. Drug Discovery Today, 14 (21-22): 1067-1074.

Bansal, T., Mishra, G., Jaggi, M., Khar, R. K., and Talegaonkar, S. 2009b. Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. European Journal of Pharmaceutical Sciences, 36(4-5): 580-590.

Betageri. G. V., 2005. Proliposomal drug delivery system. US Patent 6,849,269.

Boman, N. L., Masin, D., Mayer, L. D., Cullis, P. R., and Bally, M. B. 1994. Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors. Cancer Research, 54: 2830-2833.

Bubik, M., Ott, M., Mahringer, A., and Fricker, G. 2006. Rapid assessment of P-glycoprotein–drug interactions at the blood–brain barrier. Analytical Biochemistry, 358(1): 51-58.

Cevc, G. 1993. Phospholipids handbook. Germany: Marcel Dekker, Inc.

Chan, L. M. S., Lowes, S., and Hirst, B. H. 2004. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. European Journal of Pharmaceutical Sciences, 21(1): 25-51.

Chang, W. -K., Tai, Y. -J., Chiang, C. -H., Hu, C. -S., Hong, P. -D., and Yeh, M. -K. 2011. The comparison of protein-entrapped liposomes and lipoparticles: preparation, characterization, and efficacy of cellular uptake International Journal of Nanomedicine, 6(1)**:** 2403-2417.

Collett, A. et al. 1996. Comparison of HT29-18-C1 and Caco-2 Cell Lines as Models for Studying Intestinal Paracellular Drug Absorption. Pharmaceutical Research, 13(2)**:** 216-221.

Cuvier, C. et al. 1992. Doxorubicin-loaded nanospheres bypass tumor cell multidrug resistance. Biochemical Pharmacology, 44(3): 509-517.

Dabholkar, R. D., Sawant, R. M., Mongayt, D. A., Devarajan, P. V., and Torchilin, V. P. 2006. Polyethylene glycol-phosphatidylethanolamine conjugate (PEG-PE)-based mixed micelles: Some properties, loading with paclitaxel, and modulation of P-glycoprotein-mediated efflux. International Journal of Pharmaceutics, 315(1-2): 148-157.

Daugherty, A. L. and Mrsny, R. J. 1999. Regulation of the intestinal epithelial paracellular barrier. Pharmaceutical Science & Technology Today, 2(7): 281-287.

D'Emanuele, A., Jevprasesphant, R., Penny, J., and Attwood, D. 2004. The use of a dendrimer-propanolol prodrug to bypass efflux transporters and enhance oral bioavailability. Journal of Controlled Release, 95(3): 447-453.

Deshmukh, D. D., Ravis, W. R., and Betageri, G. V. 2008. Improved delivery of cromolyn from oral proliposomal beads. International Journal of Pharmaceutics, 358(1-2): 128-136.

De Vrueh, R. L. A., Smith, P. L., and Lee, C. -P. 1998. Transport of l-Valine-Acyclovir Via the Oligopeptide Transporter In The Human Intestinal Cell Line, Caco-2. Journal of Pharmacology and Experimental Therapeutics, 286(3)**:** 1166-1170.

Eneroth, A. et al. 2001. Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. European Journal of Pharmaceutical Sciences, 12(3)**:** 205-214.

Engdal, S. and Nilsen, O. G. 2008. Inhibition of P-glycoprotein in Caco-2 cells: Effects of herbal remedies frequently used by cancer patients. Xenobiotica, 38(6): 559-573.

Faassen, F., Vogel, G., Spanings, H., and Vromans, H. 2003. Caco-2 permeability, P-glycoprotein transport ratios and brain penetration of heterocyclic drugs. International Journal of Pharmaceutics, 263(1-2):113-122.

Fang, J. -Y., Hwang, T. -L., Huang, Y. -L., and Fang, C. -L. 2006. Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol. International Journal of Pharmaceutics, 310(1-2)**:** 131-138.

Fardel, O., Lecureur, V., and Guilouzo, A. 1996. The P-glycoprotein multidrug transporter. General Pharmacology: The Vascular System, 27(8): 1283-1291.

Feng, B. et al. 2008. In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. Drug Metabolism and Disposition, 36(2)**:** 268-275.

Föger, F., Schmitz, T., and Bernkop-Schnürch, A. 2006. In vivo evaluation of an oral delivery system for P-gp substrates based on thiolated chitosan. Biomaterials, 27: 4250-4255.

Franco, M. et al. 2008. Frog intestinal sac as an *in vitro* method for the assessment of intestinal permeability in humans: Application to carrier transported drugs. International Journal of Pharmaceutics, 352: 182-188.

Freshney, R. I. 2005. Culture of animal cells: A manual of basic technique. 5th ed. USA: Wiley-Liss.

Gillet, A., Lecomte, F., Hubert, P., Ducat, E., Evrard, B., and Piel, G. 2011. Skin penetration behaviour of liposomes as a function of their composition. European Journal of Pharmaceutics and Biopharmaceutics, 79(1)**:** 43-53.

Gochoco, C. H., Ryan, F. M., Miller, J., Smith, P. L., and Hidalgo, I. J. 1994. Uptake and transepithelial transport of the orally absorbed cephalosporin cephalexin, in the human intestinal cell line, Caco-2. International Journal of Pharmaceutics, 104: 187-202.

Goren, D., Horowitz, A. T., Tzemach, D., Tarshish, M., Zalipsky, S., and Gabizon, A. 2000. Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. Clinical Cancer Research, 6(5)**:** 1949-1957.

Gustavo, V. B. -C. 2005. Food powders: Physical properties, processing, and functionality, New York: Kluwer Academic/Plenum Publishers.

Hayashi, M., Tomita, M., and Awazu, S. 1997. Transcellular and paracellular contribution to transport processes in the colorectal route. Advanced Drug Delivery Reviews, 28(2): 191-204.

Hayeshi, R. et al. 2008. Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. European Journal of Pharmaceutical Sciences, 35: 383-396.

Hiremath, P. S., Soppimath, K. S., and Betageri, G. V. 2009. Proliposomes of exemestane for improved oral delivery: Formulation and *in vitro* evaluation using PAMPA, Caco-2 and rat intestine. International Journal of Pharmaceutics, 380(1-2): 96-104.

Hochman, J. H., Chiba, M., Yamazaki, M., Tang, C., and Lin, J. H. 2001. P-glycoprotein-mediated efflux of indinavir metabolites in Caco-2 cells expressing Cytochrome P450 3A4. The Journal of Pharmacology and Experimental Therapeutics, 298(1)**:** 323-330.

Hunter, J., Hirst, B. H., and Simmons, N. L. 1993. Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. Pharmaceutical Research, 10(5)**:** 743-749.

Huwyler, J., Cerletti, A., Fricker, G., Eberle, A. N., and Drewe, J. 2002. By-passing of P-glycoprotein using immunoliposomes. Journal of Drug Targeting, 10(1)**:** 73-79.

Hwang, B. -Y., Jung, B. -H., Chung, S. -J., Lee, M. -H., and Shim, C. -K. 1997. In vitro skin permeation of nicotine from proliposomes. Journal of Controlled Release, 49: 177-184.

Ing-orn Prasanchaimontri. 2009. Mechanism of liposomes in the delivery of P-glycoprotein substrates into Caco-2 cells. Master's thesis. Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Jain, R., Agarwal, S., Majumdar, S., Zhu, X., Pal, D., and Mitra, A. 2005. Evasion of P-gp mediated cellular efflux and permeability enhancement of HIV-protease inhibitor saquinavir by prodrug modification. International Journal of Pharmaceutics, 303(1-2): 8-19

Janga, K. Y. et al. 2011. Bioavailability enhancement of zaleplon via proliposomes: Role of surface charge. European Journal of Pharmaceutics and Biopharmaceutics, 80(2)**:** 347-357.

Jung, B. H., Chung, B. C., Chung, S. -J., Lee, M. -H., and Shim, C. -K. 2000a. Prolonged delivery of nicotine in rats via nasal administration of proliposomes. Journal of Controlled Release, 66 (1): 73-79.

Jung, T., Kamm, W., Breitenbach, A., Kaiserling, E., Xiao, J. X., and Kissel, T. 2000b. Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? European Journal of Pharmaceutics and Biopharmaceutics, 50 (1)**:** 147-160.

Junping, W., Maitani, Y., Takayama, K., and Nagai, T. 2000. In vivo evaluation of doxorubicin carried with long circulating and remote loading proliposome. International Journal of Pharmaceutics, 203(1-2): 61-69.

Kang, D. I., Kang, H. -K., Gwak, H. -S., Han, H. -K., and Lim, S. -J. 2009. Liposome composition is important for retention of liposomal rhodamine in P-glycoprotein-overexpressing cancer cells. Drug Delivery, 16(5)**:** 261-267.

Karlsson, J., Ungell, A. -L., Gråsjö, J., and Artursson, P. 1999. Paracellular drug transport across intestinal epithelia: influence of charge and induced water flux. European Journal of Pharmaceutical Sciences, 9(1): 47-56.

Kartare, O. P.,Vyas, S. P., and Dixit, V. K. 1991. Proliposomes of indomethacin for oral administration. Journal of Microencapsulation, 8(1)**:** 1-7.

Kerns, E. H. and Di, L. 2008. Chapter 5-Lipophilicity. In Drug-like properties: Concepts, structure design and methods, pp. 43-47. San Diego, Academic Press.

Kinnear, P. R. and Gray, C. D. 2011. IBM SPSS Statistic 18: Made simple. Canada: Psychology Press.

Kirby, C., Clarke, J., and Gregoriadis, G. 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and *in vitro*. Biochemical Journal, 186(2)**:** 591-598.

Knepp, V. M., Szoka Jr., F. C., and Guy, R. H. 1990. Controlled drug release from a novel liposomal delivery system. II. Transdermal delivery characteristics. Journal of Controlled Release, 12(1)**:** 25-30.

Kobayashi, T., Ishida, T., Okasa, Y., Ise, S., Harashima, H., and Kiwada, H., 2007. Effect of transferring receptor-targeted liposomal doxorubicin in P-glycoprotein-mediated drug resistant tumor cells. International Journal of Pharmaceutics, 329(1-2): 94-102.

Krieger, M. L., Konold, A., Wiese, M., Jaehde, U., and Bendas, G. 2010. Targeted doxorubicin-liposomes as a tool to circumvent P-gp-mediated resistance in ovarian carcinoma cells. International Journal of Clinical Pharmacology and Therapeutics, 48(7)**:** 442-444.

Krishna, R. and Mayer, L. D. 2000. Multidrug resistance (MDR) in cancer: Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. European Journal of Pharmaceutical Sciences, 11(4)**:** 265-283.

Lacy, C. F., Armstrong, L. L., Goldman, M. P., and Lance, L. L. 2005 Drug information handbook international with Canadian and international drug monographs, pp. 44-47. 13th edition.Hudson: Lexi-Comp Inc.

Lee, W. and Kim, R.B. 2004. Transporter and renal drug elimination. Annual Review of Pharmacology and Toxicology, 44: 137-166.

Li, X. et al. 2011. Novel mucus-penetrating liposomes as a potential oral drug delivery system: preparation, in vitro characterization, and enhanced cellular uptake. International Journal of Nanomedicine, 6: 3151-3162.

Lindenberg, M., Kopp, S., and Dressman, J.B., 2004. Classification of orally administered drugs on the World Health Organization Model list of Essential Medicines according to the biopharmaceutics classification system. European Journal of Pharmaceutics and Biopharmaceutics, 58(2): 265-278.

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews, 23(1-3): 3-25.

Lo, Y. -L, Liu, F. -I., and Cherng, J. -Y. 2001. Effect of PSC 833 liposomes and intralipid on the transport of epirubicin in Caco-2 cells and rat intestines. Journal of Controlled Released, 76(1-2): 1-10.

Luo, S., Pal, D., Shah, S. J., Kwatra, D., Paturi, K. D., and Mitra, A. K. 2010. Effect of HEPES buffer on the uptake and transport of P-glycoprotein substrates and large neutral amino acids. Molecular Pharmaceutics, 7(2)**:** 412-420.

Mamot, C., Drummond, D. C., Hong, K., Kirpotin, D. B., and Park, J. W. 2003. Liposome-based approaches to overcome anticancer drug resistance. Drug Resistance Updates, 6(5): 271-279.

Meng, M. et al. 2008. Increase of the pharmacological and pharmacokinetic efficacy of negatively charged polypeptide recombinant hirudin in rats via parenteral route by association with cationic liposomes. Journal of Controlled Release, 128(2)**:** 113-119.

Molecular Probes®. 2010. Acetomethyl (AM) and acetate esters. Invitrogen™.

Mo, R. et al. 2011. The mechanism of enhancement on oral absorption of paclitaxel by N-octyl-O-sulfate chitosan micelles. Biomaterials, doi: 10.1016/j.biomaterials.2011.03.005.

Mui, B. and Hope M. J. 2007. Formation of large unilamella vesicles by extrusion. in Gregoriadis, G, Liposome Technology vol.I: Liposome preparation and related techniques, 55-65. Florida: CRC Press Inc.

Mukaizawa, F. et al. 2009. Novel oral absorption system containing polyamines and bile salts enhances drug transport via both transcellular and paracellular pathways across Caco-2 cell monolayers. International Journal of Pharmaceutics, 367(1-2): 103-108.

Nassar, T., Rom, A., Nyska, A., and Benita, S. 2009. Novel double coated nanocapsules for intestinal delivery and enhanced oral bioavailability of tacrolimus, a P-gp substrate drug. Journal of Controlled Release, 133(1): 77-84.

New, R. R. C. 1989. Liposomes: A practical approach. New York: Oxford University Press Inc.

Ning, M. -Y., Guo, Y. -Z., Pan, H. -Z., Yu, H. -M., and Gu, Z. -W. 2005. Preparation and evaluation of proliposomes containing clotrimazole. Chemical & Pharmaceutical Bulletin, 53(6): 620-624.

Ozben, T. 2006. Mechanisms and strategies to overcome multiple drug resistance in cancer. FEBS Letters, 580(12): 2903-2909.

Palmberger, T. F., Hombach, J., and Bernkop-Schnürch, A. 2008. Thiolated chitosan: Development and in vitro evaluation of an oral delivery system for acyclovir. International Journal of Pharmaceutics, 348: 54-60.

Pappenheimer, J. 1987. Physiological regulation of transepithelial impedance in the intestinal mucosa of rats and hamster. Journal of Membrane Biology, 100(1): 137-148.

Park, S. -J., Choi, S. G., Davaa, E., and Park, J. -S. 2011. Encapsulation enhancement and stabilization of insulin in cationic liposomes. International Journal of Pharmaceutics, 415(1-2)**:** 267-272.

Patil, Y., Sadhukha, T., Ma, L., and Panyam, J. 2009. Nanoparticle-mediated simultaneous and targeted delivery of paclitaxel and tariquidar overcomes tumor drug resistance. Journal of Controlled Release, 136(1)**:** 21-29.

Payne, N. I., Timmins, P., Ambrose, C. V., and Ward, M. D. 1986. Proliposomes: A novel solution to an old problem. Journal of Pharmaceutical Sciences, 75(4) 325-329.

Pendse, S. S.,Briscoe, D. M., and Frank, M. H. 2003. P-glycoprotein and alloimmune T-cell activation. Clinical and Applied Immunology Reviews, 4(1)**:** 3-14.

Perrin, D. D. and Dempsey, B. 1974. Buffers for pH and metal ion control. Great Britain: Flesher & Son Ltd, Norwich

Riganti, C. et al. 2011. Liposome-Encapsulated Doxorubicin Reverses Drug Resistance by Inhibiting P-Glycoprotein in Human Cancer Cells. Molecular Pharmaceutics, 8(3)**:** 683-700.

Rowe, R. C., Sheskey, P. J., and Owen, S. C. 2006. Handbook of pharmaceutical excipients. 5th edition. London: Pharmaceutical Press.

Rowland, R. N. and Woodley, J. F. 1980. The stability of liposomes in vitro to pH, bile salts and pancreatic lipase. Biochimica et Biophysica Acta, 620: 400-409.

Sadava, D., Coleman, A., and Kane, S. E. 2002. Liposomal daunorubicin overcomes drug resistance in human breast, ovarian and lung carcinoma cells. Journal of Liposome Research, 12(4)**:** 301-309.

Sahay, G., Alakhova, D. Y., and Kabanov, A. V. 2010. Endocytosis of nanomedicines. Journal of Controlled Release, 145(3)**:** 182-195.

Salama, N. N., Scott, K. R., and Eddington, N. D. 2004. DM27, an enaminone, modifies the in vitro transport of antiviral therapeutic agents. Biopharmaceutics & Drug disposition, 25: 227-236.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., and Zucco, F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biology and Toxicology, 21(1)**:** 1-26.

Schinkel, A. H. and Jonker, J. W. 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Advanced Drug Delivery Reviews, 55(1): 3-29.

Shah, P., Jogani, V., Bagchi, T., and Misra, A. 2006. Role of caco-2 cell monolayers in prediction of intestinal drug absorption. Biotechnology Progress, 22: 186-198.

Shargel, L., Wu-Pong, S., and Yu, A. B. C. 2005. Applied Biopharmaceutics & Pharmacokinetics. New York: McGraw-Hill.

Sharom, F. J. 2008. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics, 9(1)**:** 105-127.

Sheue Nee Ling, S., Magosso, E., Abdul Karim Khan, N., Hay Yuen, K., and Anne Barker, S. 2006. Enhanced Oral Bioavailability and Intestinal Lymphatic Transport of a Hydrophilic Drug Using Liposomes. Drug Development and Industrial Pharmacy, 32(3)**:** 335-345.

Song, C. K, Balakrishnan, P., Shim, C. -K. Chung, S. -J., and Kim, D. -D. 2011. Enhanced in vitro cellular uptake of P-gp substrate by poloxamer-modified liposomes (PMLs) in MDR cancer cells. Journal of Microencapsulation, 28(6): 575-581.

Song, K. -H., Chung, S. -J., and Shim, C. -K. 2002. Preparation and evaluation of proliposomes containing salmon calcitonin. Journal of Controlled Release, 84(1-2): 27-37.

Song, K. -H., Chung, S. -J., and Shim, C. -K. 2005. Enhanced intestinal absorption of salmon calcitonin (sCT) from proliposomes containing bile salts. Journal of Controlled Release, 106(3): 298-308.

Straubinger, R. M., Hong, K., Friend, D. S., and Papahadjopoulos, D. 1983. Endocytosis of liposomes and intracellular fate of encapsulated molecules: Encounter with a low pH compartment after internalization in coated vesicles. Cell, 32(4)**:** 1069-1079.

Szoka Jr., F. C. and Papahadjopoulos, D. 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annual Review of Biophysics and Bioengineering, 9: 467-508.

Takano, M., Yumoto, R., and Murakami, T. 2006. Expression and function of efflux drug transporters in the intestine. Pharmacology & Therapeutics, 109(1-2): 137-161.

Takeda, M. et al. 2002. Human organic anion transporters and human organic cation transporters mediate renal antiviral transport. The Journal of Pharmacology and Experimental Therapeutics, 300(3)**:** 918-924.

Taubert, D. et al. 2006. Impact of P-gp on clopidogrel absorption. Clinical Pharmacology & Therapeutics, 80: 486-501.

Taylor, M. D. 1996. Improved passive oral drug delivery via prodrugs. Advanced Drug Delivery Reviews, 19(2): 131-148.

The United States Pharmacopeia Convention. 2012. The United States Pharmacopoeia 35 and the National Formulary 30. Washington D.C.

Troutman, M. D. and Thakker, D. R. 2003. Rhodamine 123 Requires Carrier-Mediated Influx for Its Activity as a P-Glycoprotein Substrate in Caco-2 Cells. Pharmaceutical Research, 20(8)**:** 1192-1199.

Ungell, A. -L. and Abrahamsson, B. 2001. Biopharmaceutical support in candidate drug selection. in M. Gibson (ed.), Pharmaceutical preformulation and formulation: A practical guide from candidate drug selection to commercial dosage form, pp. 120-121. Englewood: IHS® Health Group.

U.S. Food and Drug Administration. 2011. Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers [online]. Available from: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm [2012, April 19]

U.S. Food and Drug Administration. 2012. Drugs@FDA: FDA Approved Drug Products [online]. Available from: http://www.accessdata.fda.gov/drugsatfda_docs /label/2004/18603slr 027 _ zovirax_lbl.pdf [2012, April 19]

Utoguchi, N., Chandorkar, G. A., Avery, M., and Audus, K. L. 2000. Functional expression of P-glycoprotein in primary cultures of human cytotrophoblasts and BeWo cells. Reproductive Toxicology, 14(3)**:** 217-224.

Varma, M. V. S., Ashokraj, Y., Dey, C. S., and Panchagnula, R. 2003. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacological Research, 48(4): 347-359.

Varma, M. V. S., Perumal, O. P., and Panchagnula, R. 2006. Functional role of P-glycoprotein in limiting peroral drug absorption: optimizing drug delivery. Current Opinion in Chemical Biology, 10(4)**:** 367-373.

Wahlang, B., Pawar, Y. B., and Bansal, A. K. 2011. Identification of permeability-related hurdles in oral delivery of curcumin using the Caco-2 cell model. European Journal of Pharmaceutics and Biopharmaceutics, 77(2): 275-282.

Walter, E., Kissel, T., and Amidon, G. L. 1996. The intestinal peptide carrier: A potential transport system for small peptide derived drugs. Advanced Drug Delivery Reviews, 20(1)**:** 33-58.

Wang, E. -J., Casciano, C. N., Clement, R. P., and Johnson, W. W. 2000. Cholesterol Interaction with the Daunorubicin Binding Site of P-Glycoprotein. Biochemical and Biophysical Research Communications, 276(3)**:** 909-916.

Washington, N., Washington, C., and Wilson, C. G. 2001. Physiological pharmaceutics: Barriers to drug absorption. 2nd ed. Great Britain: TJ International Ltd.

Wilschut, J. 1982. in Leser, D., and Barbet, J., Preparation and properties of phospholipid vesicles, 10-24.

Xia, F., Hu, D., Jin, H., Zhao, Y., and Liang, J. 2010. Preparation of lutein proliposomes by supercritical anti-solvent technique. Food Hydrocolloids, doi: 10.1016/j.foodhyd.2010.11.014.

Xu, H., He, L., Nie, S., Guan, J., Zhang, X., Yang, X., and Pan, W. 2009. Optimized preparation of vinpocetine proliposomes by a novel method and in vivo evaluation of its pharmacokinetics in New Zealand rabbits. Journal of Controlled Release, 140(1): 61-68.

Yan-Yu, X., Yun-Mei, S., Zhi-Peng, C., and Qi-Neng, P. 2006. Preparation of silymarin proliposome: A new way to increase oral bioavailability of silymarin in beagle dogs. International Journal of Pharmaceutics, 319(1-2):162-168.

Zastre, J., Jackson, J., Bajwa, M., Liggins, R., Iqbal, F., and Burt, H. 2002. Enhanced cellular accumulation of a P-glycoprotein substrate, rhodamine-123, by caco-2

cells using low molecular weight methoxypolyethylene glycol-block-polycaprolactone diblock copolymers. European Journal of Pharmaceutics and Biopharmaceutics, 54(3)**:** 299-309.

Zhao-Gang, Y., Huan, M., Xuan, Z., Xiao-Dan, L., Wan-Liang, L., and Qiang, Z. 2004. Effect of quercetin on the acyclovir intestinal absorption. Journal of Peking University (Health Sciences), 36(3)**:** 309-312.

APPENDICES

APPENDIX A

Particle sizes of sorbitol particles and sorbitol-acyclovir particles

Table A1: Particle sizes of sorbitol particles and sorbitol-acyclovir particles used as carrier for preparation of proliposomes

Particle size (µm)	Sorbitol (%)	Sorbitol-acyclovir (%)
< 150	24.74, 24.69	21.41, 23.35
150-425	75.26, 75.31	78.59, 76.65

APPENDIX B

Modified simulated intestinal fluid (mSIF)

Modified simulated intestinal fluid

HEPES	11.92	g
NaOH	0.30	g
NaCl	0.43	g
adjust pH to 6.8 with NaOH		
Water to	1000	ml

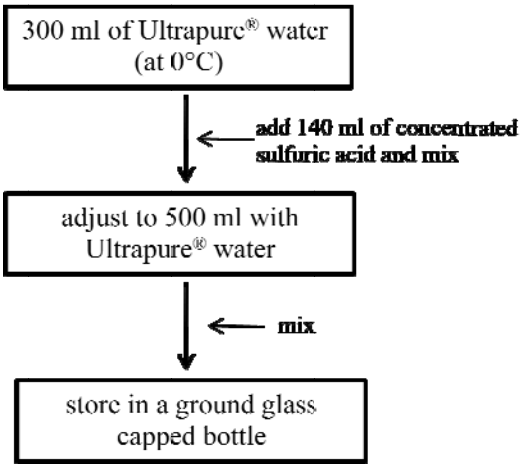
APPENDIX C

Bartlett assay (New, 1989)

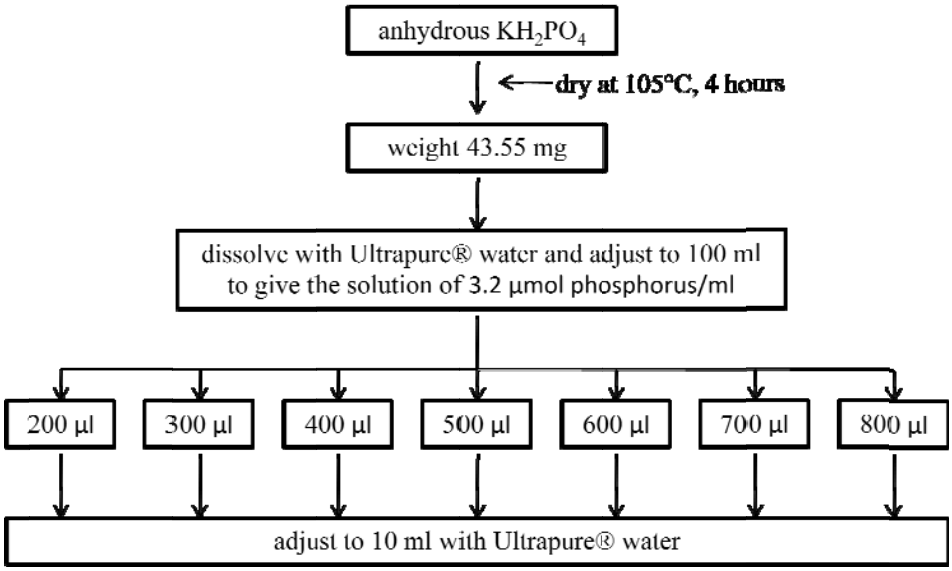
Protocol for analysis of phosphorus using the Bartlett assay

(A) Preparation of reagents

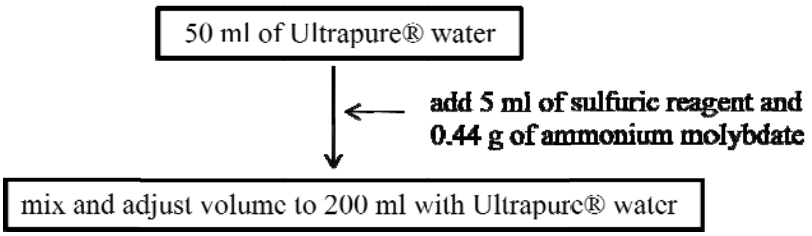
- *Sulfuric acid (5M) reagent*



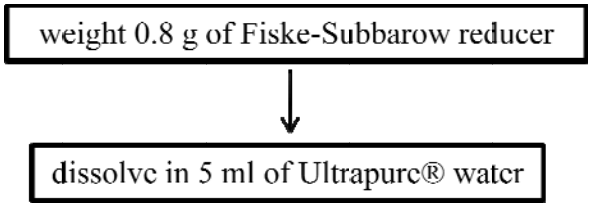
- *Phosphate standard solutions*



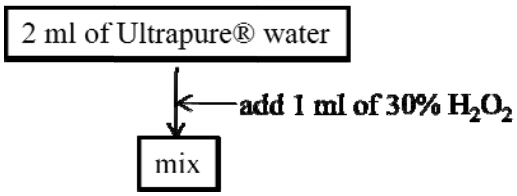
- *Ammonium molybdate–sulfuric acid solution (Acid-molybdate)*



- *1-amino-2-naphthol-4-sulfonic acid reagent (Fiske & Subbarow reagent)*



- *Hydrogen peroxide (H_2O_2)*



(B) **Sample preparation**

Dilute the liposome dispersion with Ultrapure® water to give a concentration of SPC of approximately 1 mg/ml

(C) **Assay method**

Figure C1: A representation of standard calibration lines for phosphatidylcholine determination ($y = 2.4701x-0.0017$, $R^2=0.9985$)

APPENDIX D

Validation of acyclovir analysis by the UV spectrophotometric method

1. Specificity

Figure D1 shows the UV spectra at 254 nm of acyclovir working standard, proliposomes and acyclovir-loaded proliposomes with SPC:CHO ratio of 1:0 and 1:0.25, respectively.

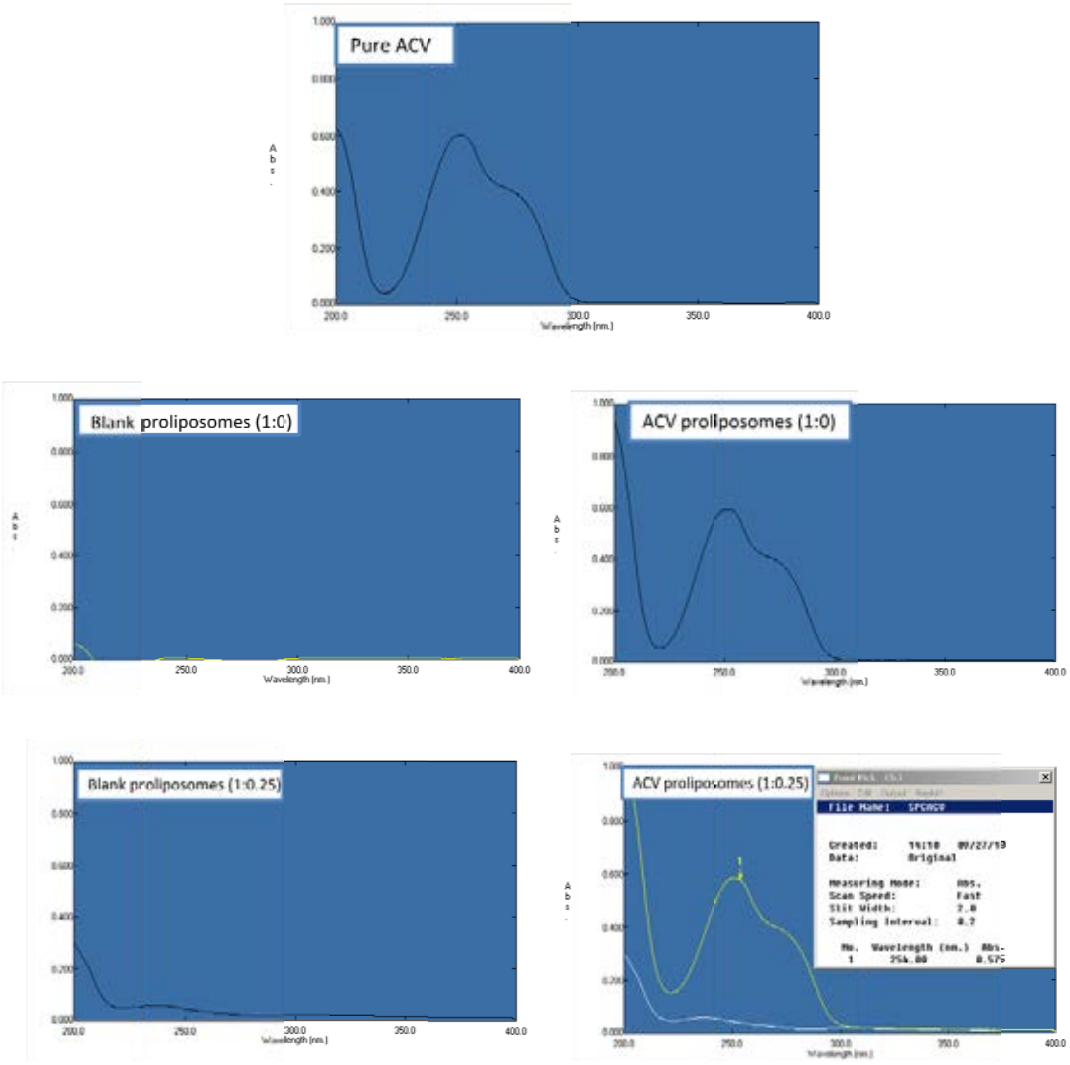


Figure D1: UV spectra of acyclovir working standard, blank proliposomes and acyclovir-loaded proliposomes with SPC:CHO ratios of 1:0 and 1:0.25

2. Linearity

Data of the working standard acyclovir at various concentrations and the corresponding absorbance intensities are shown in Table D1. The plot of a calibration line is depicted in Figure D2. The plot shows good linearity with R^2 in the range of 0.9999-1.0000.

Table D1: Data for the calibration lines of acyclovir in Ultrapure® water by the UV spectrophotometric method

Concentration (µg/ml)	Absorbance			Mean	SD	%CV
	Set 1	Set 2	Set 3			
4	0.2295	0.2294	0.2276	0.2288	0.0011	0.48
7	0.3991	0.3936	0.3949	0.3959	0.0029	0.73
10	0.5675	0.5648	0.5635	0.5653	0.0020	0.35
13	0.7333	0.7321	0.7311	0.7322	0.0011	0.15
16	0.8983	0.8944	0.8978	0.8968	0.0021	0.23
R^2	1.0000	0.9999	1.0000	1.0000	-	-

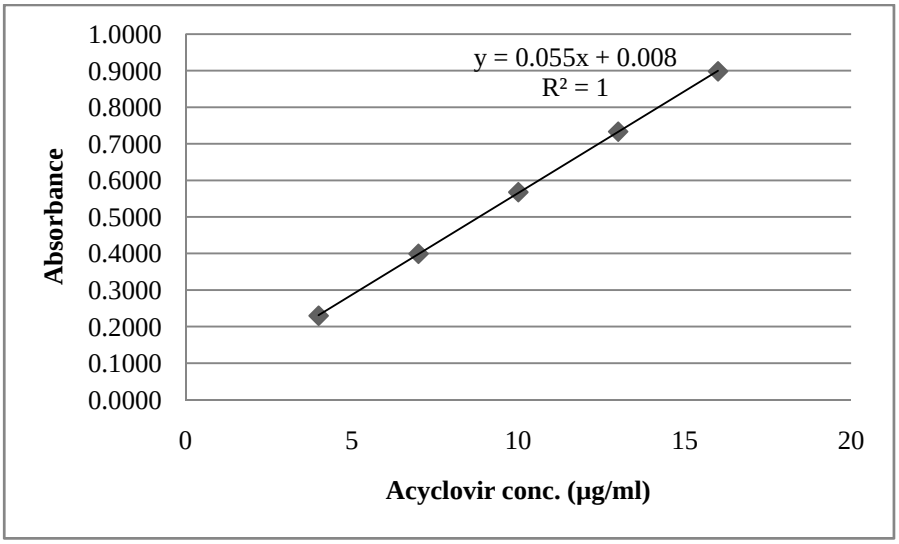


Figure D2: A representation of the calibration lines of acyclovir in Ultrapure water® by the UV spectrophotometric method

3. Accuracy

The percentages of analytical recovery of acyclovir are shown in Tables D2 and D3. The percentages of analytical recovery of acyclovir are in the acceptable range (±10%) indicating that this method could be used for acyclovir analysis at all concentrations within the studied range with satisfactory accuracy.

Table D2: The percentages of analytical recovery of acyclovir in Ultrapure® water by the UV spectrophotometric method (SPC:CHO ratio of 1:0)

Concentration (µg/ml)	**Analytical recovery (%)**			**Mean ± SD**
	1:0 of SPC:CHO			
	1	**2**	**3**	
5	101.54	100.75	101.13	101.14 ± 0.40
8	98.86	99.24	98.74	98.95 ± 0.26
11	99.72	100.58	99.44	99.91 ± 0.59
14	98.82	98.99	98.90	98.90 ± 0.09

Table D3: The percentages of analytical recovery of acyclovir in Ultrapure® water by the UV spectrophotometric method (SPC:CHO ratio of 1:0.25)

Concentration (µg/ml)	**Analytical recovery (%)**			**Mean ± SD**
	1:0.25 of SPC:CHO			
	1	**2**	**3**	
5	98.77	101.06	99.71	99.85 ± 1.15
8	98.27	97.61	99.02	98.30 ± 0.70
11	100.17	99.40	98.32	99.29 ± 0.93
14	98.91	100.09	98.01	99.00 ± 1.04

4. Precision

The precision of acyclovir analyzed by the UV spectrophotometric method were determined for both the within run precision and the between run precision as illustrated in Tables D4 and D5. The coefficient of variation values were 0.24-1.41 and 0.10-0.89, respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the UV spectrophotometric method was sufficiently precise for the quantitative analysis of acyclovir in the concentration range studied.

Table D4: Data of the within run precision of acyclovir in Ultrapure® water by the UV spectrophometric method

Concentration (µg/ml)	**Concentration (µg/ml)**			**Mean ± SD**	**%CV**
	1	**2**	**3**		
5	5.26	5.27	5.39	5.30 ± 0.08	1.41
8	8.42	8.45	8.52	8.46 ± 0.05	0.62
11	11.68	11.74	11.70	11.71 ± 0.03	0.26
14	14.80	14.84	14.77	14.80 ± 0.04	0.24

Table D5: Data of the between run precision of acyclovir in Ultrapure® water by the UV spectrophometric method

Concentration (µg/ml)	**Concentration (µg/ml)**			**Mean ± SD**	**%CV**
	1	**2**	**3**		
5	5.26	5.27	5.33	5.28 ± 0.04	0.75
8	8.42	8.44	8.51	8.46 ± 0.05	0.55
11	11.68	11.57	11.78	11.68 ± 0.10	0.89
14	14.80	14.78	14.77	14.78 ± 0.01	0.10

APPENDIX E

Standard calibration lines for calcein and rhodamin123

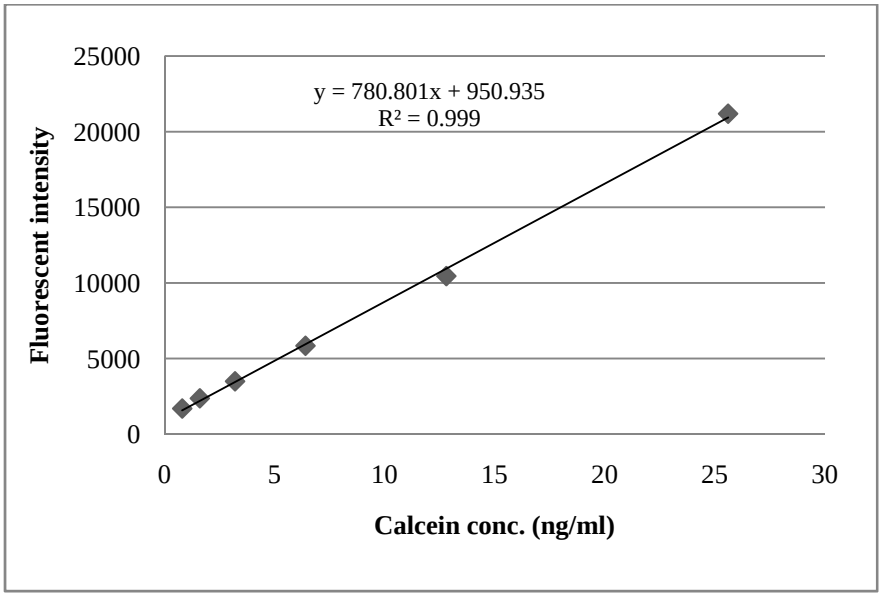


Figure E1: A representation of the standard calibration lines for calcein in 0.3 N sodium hydroxide containing 1% Triton X-100 (with cells)

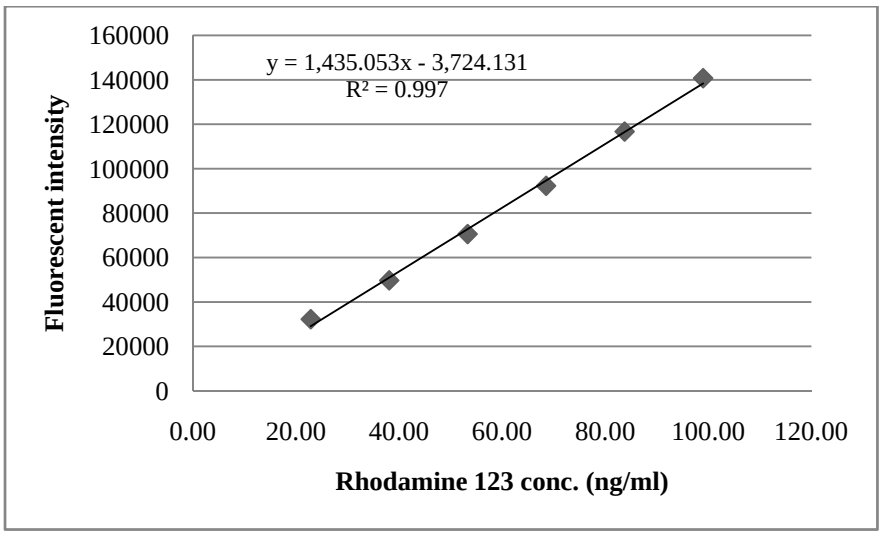


Figure E2: A representation of the standard calibration lines for rhodamine 123 in PBS containing 1% Triton X-100 (without cells)

APPENDIX F

Standard calibration lines of calcein and rhodamin123 in the presence and the absence of cells

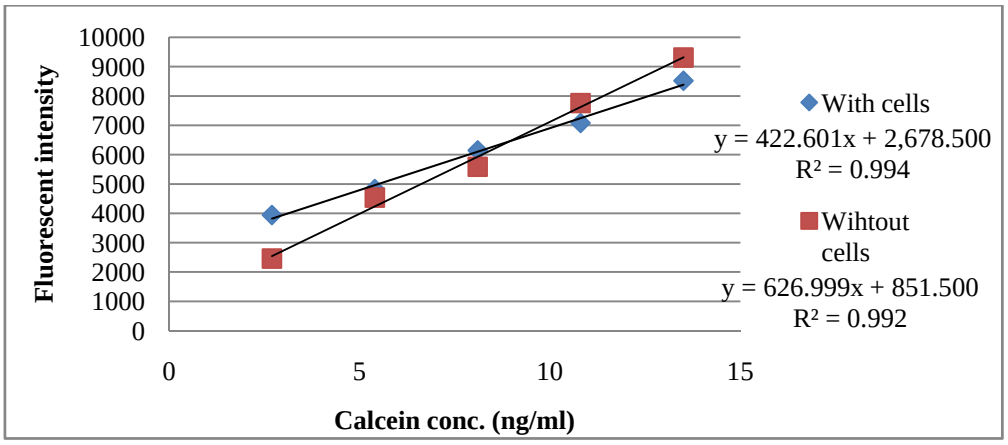


Figure F1: The standard calibration lines of calcein in 0.3 N sodium hydroxide containing 1% Triton X-100, in the presence and the absence of the Caco-2 cell lysates

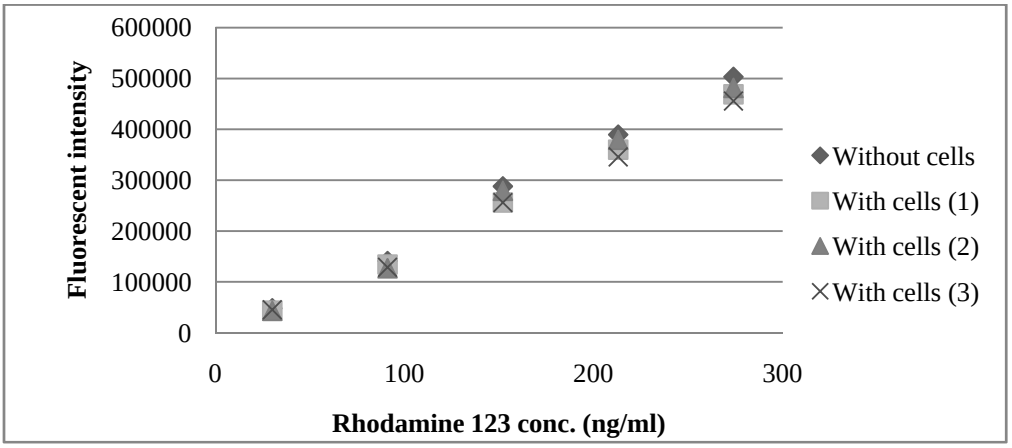


Figure F2: The standard calibration lines of rhodamine 123 in PBS containing 1% Triton X-100, in the presence and the absence of the Caco-2 cells lysates. The actual standard calibration lines used in the uptake studies were constructed in the range of 20-100 ng/ml only.

Without cells: y = 1900.613x-14989.266 R^2 = 0.996

With cells (1): y = 1766.841x-16190.219 R^2 = 0.999

With cells (2): y = 1852.670x-19811.237 R^2 = 0.993

With cells (3): y = 1702.036x-12566.025 R^2 = 0.997

APPENDIX G

Cell viability determined at the end of the uptake experiments

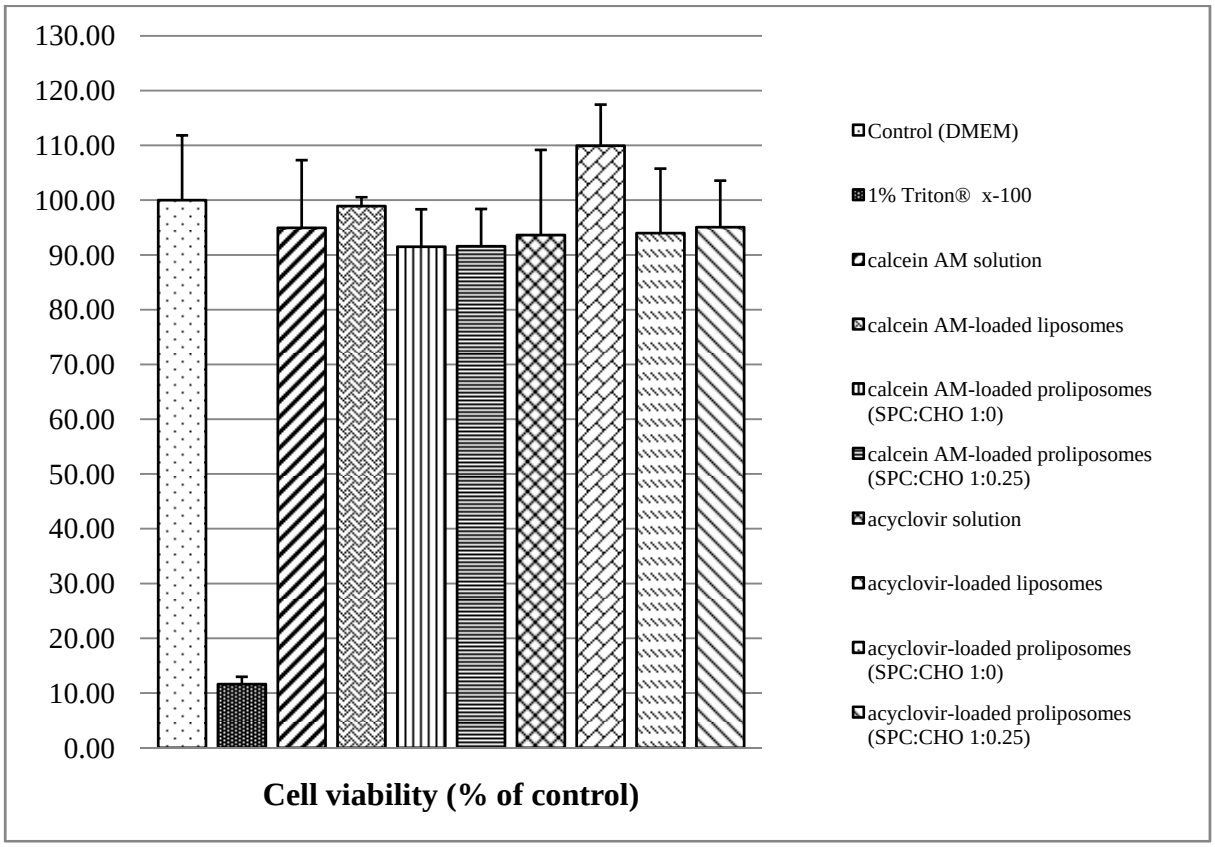


Figure G1: Viability of the cells by the MTT assay after exposure to various test solutions/dispersions. The conditions used were the same as those used in the uptake experiments. Data are shown as mean ± SD.

APPENDIX H

Validation and verification of the assay method for acyclovir determination by HPLC

Analysis of acyclovir by high performance liquid chromatographic (HPLC) method (The United States Pharmacopoeia Convention, 2012**)**

Validation of acyclovir assay method (LC-10, Shimadzu, Japan)

In this study, the suspension of the digested cells was centrifuged at 13,000 rpm (Minispin®, Eppendorf, Germany) for 5 minutes. The supernatant was filtered through a 0.45 µm of Nylon membrane and further injected in to the HLPC system, which was equipped with the 254 nm UV-detector. The column used in this study was C-18 with the column length of 15 mm. The mobile phase was the 0.1% glacial acetic acid in water and the flow rate was 2 ml/minute. The conditions used in this study were validated on the LC-10 series (Shimadzu, Japan) and further verified on the 1100 series (Agilent, Germany). The working standard of acyclovir was 99.2% in purity.

1. Specificity

Figure H1 shows typical chromatograms of the working standard of acyclovir, with and without cell lysates, in 1% Triton X-100 in PBS. The retention time of acyclovir was at 12.718 and 12.823 minutes in the absence and in the presence of cell lysates, respectively.

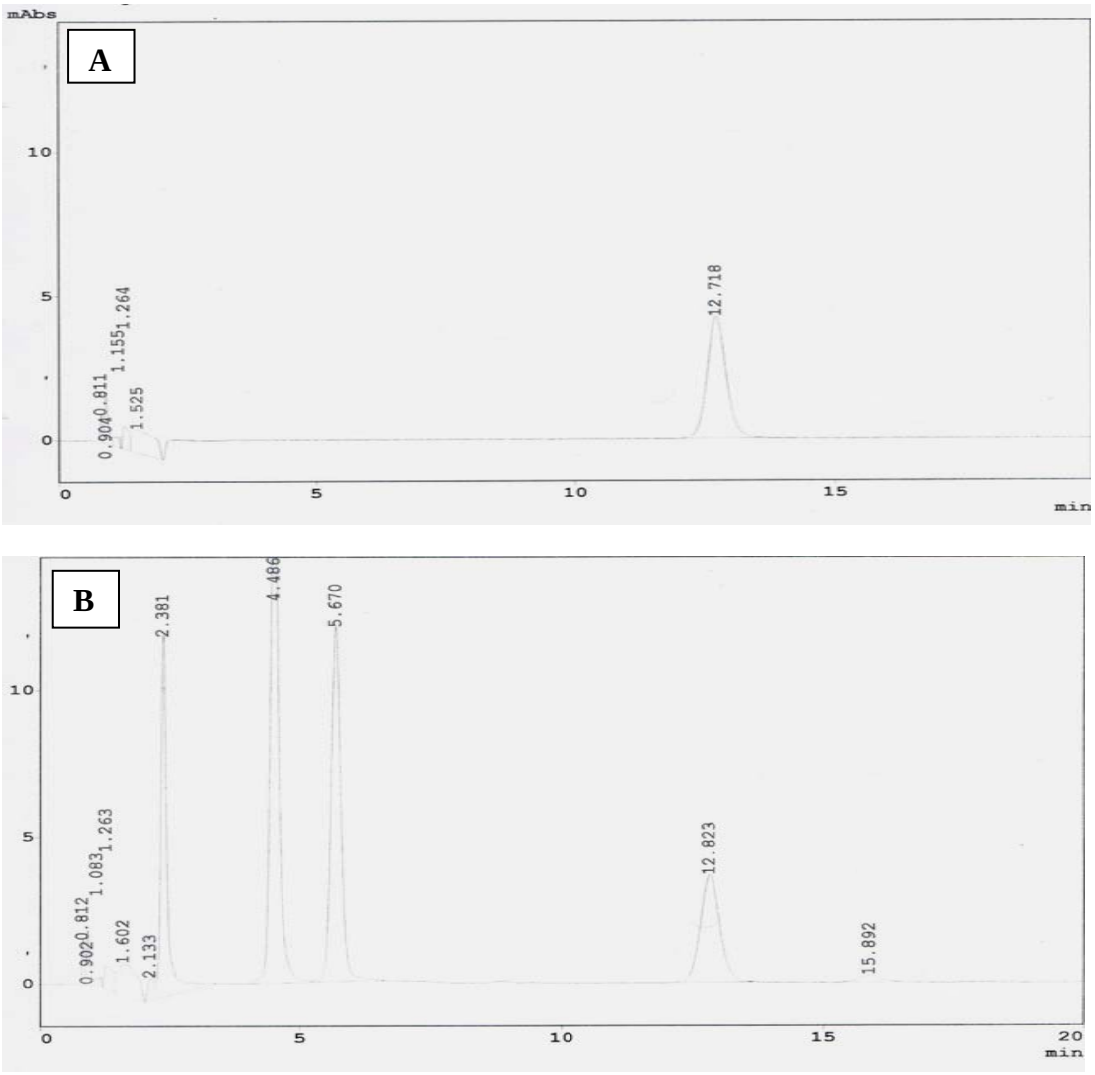


Figure H1: HPLC chromatograms of the working standard of acyclovir in 1% Triton X-100 in PBS, in the (A) absence and (B) presence of the Caco-2 cell lysates

2. Linearity

Data of concentrations and corresponding peak areas of the acyclovir working standard are shown in Table H1. A representation plot of the standard calibration lines is depicted in Figure H2. The plot shows acceptable linearity with R^2 in the range of 0.9985-0.9990.

Table H1: Data for the calibration lines of acyclovir in 1% Triton X-100 in PBS by the HPLC method

Concentration (µg/ml)	Peak area (mAUs)			Mean	SD	%CV
	Set 1	Set 2	Set 3			
1.0	28910	28396	28239	28515.00	350.97	1.23
1.6	47759	47656	47035	47483.33	391.67	0.82
2.2	61684	62274	61933	61963.67	296.19	0.48
2.8	82501	83060	82520	82693.67	317.40	0.38
3.4	99426	99332	99646	99468.00	161.16	0.16
4.0	116390	116300	116785	116491.67	257.99	0.22
R^2	0.9985	0.9987	0.9990	0.9988	-	-

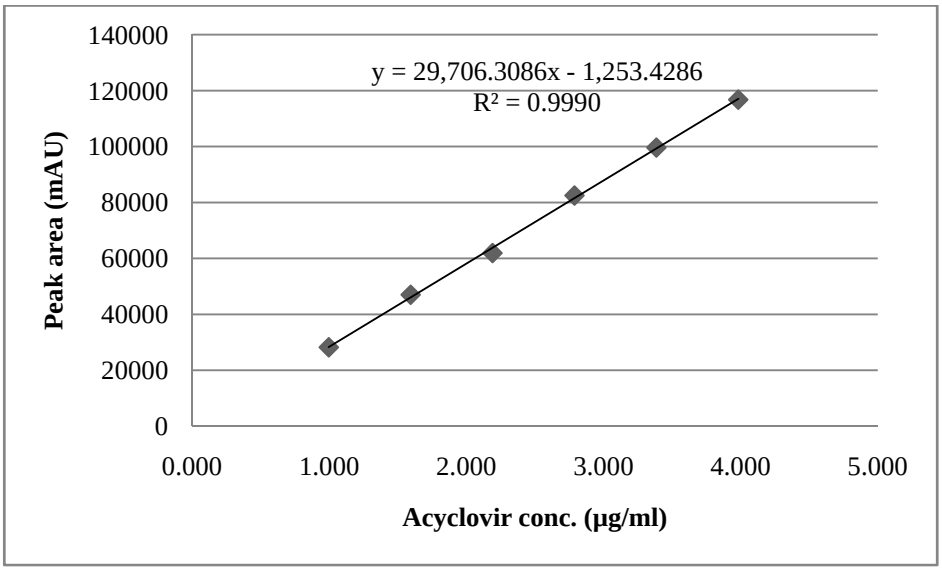


Figure H2: A representation of the calibration lines of acyclovir in 1% Triton X-100 in PBS, pH 7.4, by the HPLC method

3. Accuracy

The percentages of analytical recovery of acyclovir are shown in Table H2. The percentages of analytical recovery of acyclovir are in the acceptable range (±10%) indicating that this method could be used for acyclovir analysis at all concentrations within the studied range with satisfactory accuracy.

Table H2: The percentages of analytical recovery of acyclovir in 1% Triton X-100 in PBS, in the presence of Caco-2 cell lysates, by the HPLC method

Concentration (µg/ml)	**Analytical recovery (%)**			**Mean ± SD**
	1	**2**	**3**	
1.3	100.75	99.58	92.42	97.58 ± 4.51
2.5	96.00	94.65	92.87	94.51 ± 1.57
3.7	95.48	97.05	93.30	95.27 ± 1.88

4. Precision

The precision of acyclovir analyzed by the HPLC method were determined for both the within run precision and the between run precision as illustrated in Tables H3 and H4. The coefficient of variation values were 0.16-1.03 and 0.12-1.11, respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the HPLC method was sufficiently precise for the quantitative analysis of acyclovir in the concentration range studied.

Table H3: Data of the within run precision of acyclovir in 1% Triton X-100 in PBS by the HPLC method

Concentration (µg/ml)	**Concentration (µg/ml)**			**Mean ± SD**	**%CV**
	1	**2**	**3**		
1.3	1.41	1.38	1.41	1.40 ± 0.01	1.03
2.5	2.64	2.64	2.64	2.64 ± 0.00	0.16
3.7	3.98	3.95	3.96	3.96 ± 0.01	0.30

Table H4: Data of the between run precision of acyclovir in 1% Triton X-100 in PBS by the HPLC method

Concentration (µg/ml)	Concentration (µg/ml)			Mean ± SD	%CV
	1	2	3		
1.3	1.38	1.39	1.41	1.39 ± 0.02	1.11
2.5	2.64	2.63	2.64	2.63 ± 0.00	0.18
3.7	3.96	3.95	3.96	3.96 ± 0.00	0.12

Verification of acyclovir determination method

(The 1100 series, Agilent, Germany)

1. Accuracy

The accuracy of the analytical method on the 1100 series was further verified because the LC-10 series was out of order and the assay had to be done on a different instrument. The percentages of analytical recovery of acyclovir are shown in Table H5. The percentages of analytical recovery of acyclovir are in the acceptable range (± 10%) indicating that this method could be used for acyclovir analysis at all concentrations within the studied range with satisfactory accuracy.

Table H5: The percentages of analytical recovery of acyclovir in 1% Triton X-100 in PBS, in the presence of Caco-2 cell lysates, by the HPLC method

Concentration (µg/ml)	Analytical recovery (%)			Mean ± SD
	1	2	3	
1.3	86.88	89.50	102.15	92.84 ± 8.16
2.5	83.67	95.85	95.02	91.51 ± 6.80
3.7	88.49	94.37	97.00	93.29 ± 4.36

2. Precision

The within run precision of acyclovir was also verified by the HPLC method as illustrated in Table H6. The coefficient of variation values was as 0.10-0.95. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the HPLC method was sufficiently precise for the quantitative analysis of acyclovir in the concentration range studied.

Table H6: Data of the within run precision of acyclovir in 1% Triton X-100 in PBS by the HPLC method

Concentration (µg/ml)	**Concentration (µg/ml)**			**Mean ± SD**	**%CV**
	1	**2**	**3**		
1.3	1.37	1.38	1.38	1.38 ± 0.01	0.39
2.5	2.54	2.59	2.58	2.57 ± 0.02	0.95
3.7	3.84	3.84	3.83	3.84 ± 0.00	0.10

APPENDIX I

Flow properties and corresponding angles of repose

(The United States Pharmacopoeia Convention, 2012)

Table I1: Flow properties and corresponding angles of repose (The United States Pharmacopoeia Convention, 2012)

Flow property	Angle of repose (degrees)
Excellent	25-30
Good	31-35
Fair – aid not needed	36-40
Passable – may hang up	41-45
Poor – must agitate, vibrate	46-55
Very poor	56-65
Very, very poor	> 66

APPENDIX J

Particle size distributions of the extruded liposomes and of the resultant liposomes formed from the blank proliposomes

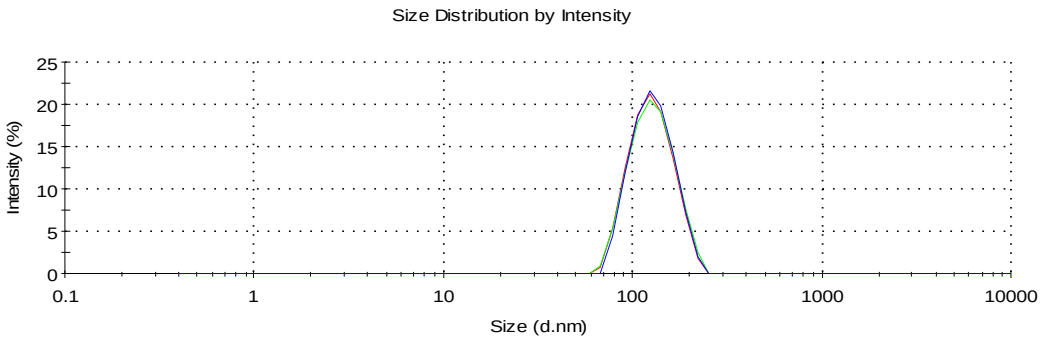


Figure J1: Particle size distribution of the extruded liposomes (Zetasizer Nano ZS, Malvern, UK)

TableJ1: Mean particle size of the extruded liposomes from three measurements

Measurement time	Particle size (nm)
1	121.4
2	122.5
3	123.1
Mean ± SD	122.33 ± 0.86

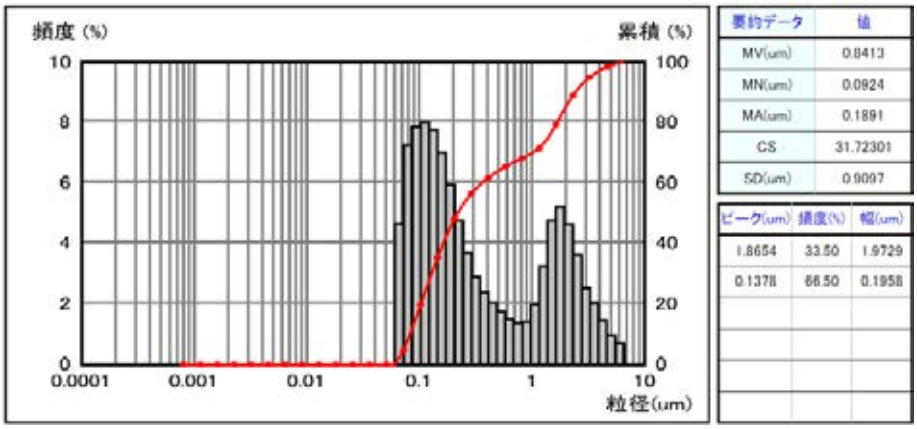


Figure J2: Particle size distribution of the resultant liposomes from the blank proliposomes (Microtrac UPA® Nikkiso, Japan; measurement range: 0.0008–6.5μm)

APPENDIX K

Analytical recoveries of acyclovir and SPC in entrapment efficiency determination

Table K1: Analytical recovery of acyclovir in entrapment efficiency determination

SPC:CHO	day	Analytical recovery* (%) (mean ± SD)		
		supernatant	pellet	total
1:0	0	83.86 ± 3.03	4.37 ±0.84	88.24 ± 3.35
	7	85.37 ± 1.82	4.37 ± 0.10	89.74 ± 1.92
	14	86.60 ± 4.60	4.43 ± 0.19	91.03 ± 4.57
1:0.25	0	80.32 ± 1.29	4.10 ± 0.37	84.42 ± 1.63
	7	88.54 ± 4.09	4.31 ± 0.71	92.86 ± 4.67
	14	84.86 ± 2.96	5.26 ± 0.62	90.12 ± 2.36

***** Based on the calculated amount of acyclovir in the corresponding proliposome preparation

Table K2: Analytical recovery of SPC in entrapment efficiency determination

SPC:CHO	day	Analytical recovery* (%) (mean ± SD)		
		supernatant	pellet	total
1:0	0	1.14 ± 0.27	81.81 ± 14.68	82.95 ± 14.89
	7	0.02 ± 0.03	81.01 ± 10.38	81.01 ± 10.38
	14	1.73 ± 0.80	81.61 ± 7.51	83.34 ± 8.01
1:0.25	0	1.00 ± 0.20	70.61 ± 2.06	80.61 ± 1.91
	7	3.18 ± 4.36	92.28 ± 2.68	95.46 ±6.92
	14	0.12 ± 0.20	89.64 ± 4.64	89.75 ± 4.64

***** Based on the calculated amount of SPC in the corresponding proliposome preparation

APPENDIX L

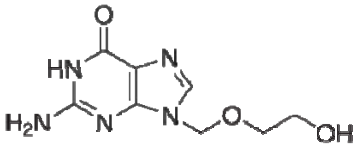
Molecular structures of acyclovir, calcein AM, calcein, cyclosporine A, rhodamine 123, and verapamil hydrochloride (Sigma-Aldrich, 2011)

Acyclovir (Sigma-Aldrich, 2011)

Synonym: 9-[(2-Hydroxyethoxy) methyl]guanine

Empirical: $C_8H_{11}N_5O_3$

Structure:



(From Sigma-Aldrich, 2011)

Molecular weight: 225.2

Solubility: 0.7 mg/ml in water, 50 mg/ml in 1M HCl, 7 mg/ml in DMSO

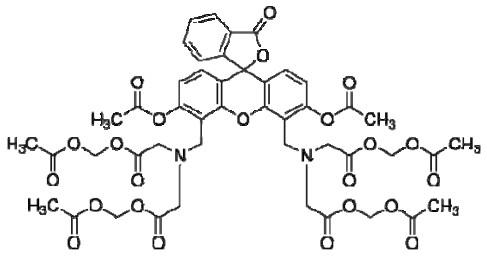
Storage temperature: store at room temperature

Calcein AM (Sigma-Aldrich, 2011)

Synonym: Calcein *O*,*O*′-diacetate tetrakis(acetoxymethyl) ester

Empirical: $C_{46}H_{46}N_2O_{23}$

Structure:



(From Sigma-Aldrich, 2011)

Molecular weight: 994.86

Solubility: soluble in DMSO

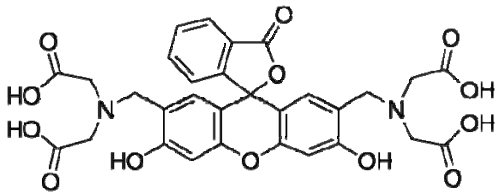
Storage temperature: −20°C

Calcein (Sigma-Aldrich, 2011)

Synonyms: Fluorescein-bis(methyliminodiacetic acid), Fluorexon, Bis[*N*,*N*−bis(carboxymethyl)aminomethyl]fluorescein

Empirical: $C_{30}H_{26}N_2O_{13}$

Structure:



(From Sigma-Aldrich, 2011)

Molecular weight: 622.53

Solubility: clear orange to brown solution at 50 mg/ml in 1 M sodium hydroxide

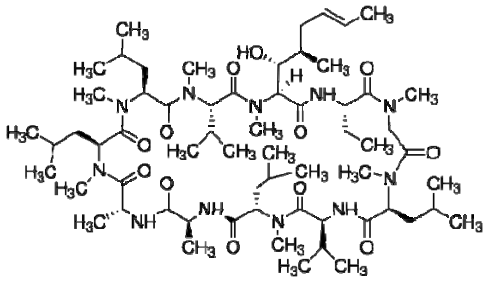
Storage temperature: store at room temperature

Cyclosporine A (Sigma-Aldrich, 2011)

Synonyms: Cyclosporine, Antibiotic S 7481F1

Empirical: $C_{62}H_{111}N_{11}O_{12}$

Structure:



(From Sigma-Aldrich, 2011)

Molecular weight: 1202.61

Solubility: soluble in DMSO, diethylether, chloroform

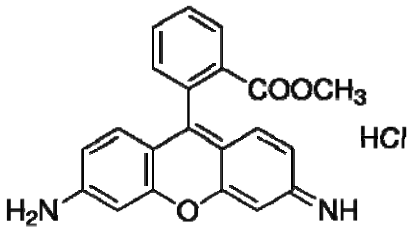
Storage temperature: −20°C

Rhodamine 123 (Sigma-Aldrich, 2011)

Synonym: 2-(6-Amino-3-imino-3H-xanthen-9-yl) benzoic acid methyl ester

Empirical: $C_{21}H_{17}ClN_2O_3$

Structure:



(From Sigma-Aldrich, 2011)

Molecular weight: 380.83

Solubility: soluble in 100% ethanol and water

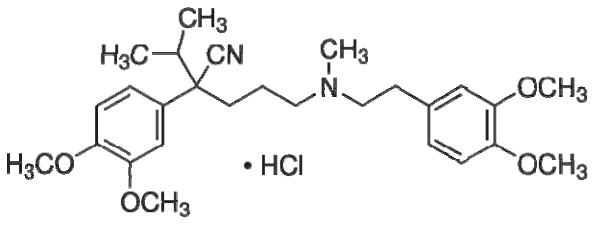
Storage temperature: store at room temperature

± Verapamil hydrochloride (Sigma-Aldrich, 2011)

Synonym: 5-[*N*-(3,4-Dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride

Empirical: $(CH_3O)_2C_6H_3CH_2CH_2N(CH_3)(CH_2)_3C[C_6H_3(OCH_3)_2][CH(CH_3)_2]CN \cdot HCl$

Structure:



(From Sigma-Aldrich, 2011)

Molecular weight: 491.06

Solubility: 50 mg/ml in water, soluble in ethanol

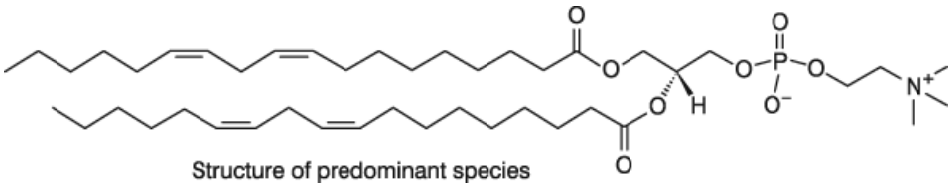
Storage temperature: store at room temperature

APPENDIX M

Molecular structures of phosphatidyl choline and cholesterol

(Avanti Polar Lipids**;** Sigma-Aldrich, 2011)

Molecular structure of phosphatidylcholine



(From Avanti Polar Lipids)

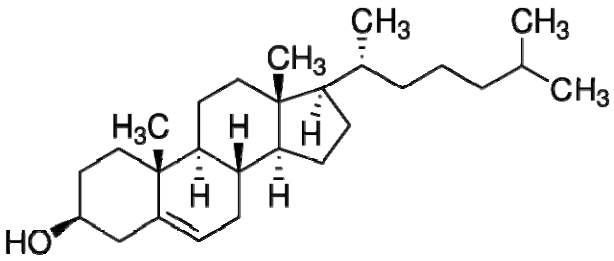
Cholesterol

Synonym: 3β-Hydroxy-5-cholestene, 5-Cholesten-3β-ol

Empirical: $C_{27}H_{46}O$

Molecular weight: 386.65

Structure:



(From Sigma-Aldrich, 2011)

VITA

Miss Kesinee Netsomboon was born on November 9, 1984 in Mae Hong Son, Thailand. She received the Bachelor of Pharmacy from Chiang Mai University in 2007. She entered the master's degree program in Pharmacy at Chulalongkorn University in 2008.