EFFECT OF PEGYLATION ON THE DELIVERY OF PHOSPHOLIPID-BASED LIPOSOMES INTO CACO-2 CELLS

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ผลของการใส่พอลิเอทิลีนไกลคอลต่อการนำส่งลิโพโซมที่เตรียมจากฟอสโฟลิพิดเข้าสู่เซลล์คาโค-2

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การศึกษานี้มีวัตถุประสงค์เพื่อประเมินผลของการใส่พอลิเอทิลีนไกลคอลต่อการนำส่งลิโพโซมที่เตรียมจากฟอสโฟลิพิดเข้าสู่เซลล์คาโค-2 เปรียบเทียบกับลิโพโซมแบบดั้งเดิม รวมถึงศึกษาผลของการใส่ประจุบนพื้นผิวลิโพโซม นอกจากนี้ยังศึกษาถึงกลไกหลักในการนำส่งลิโพโซมที่มีพอลิเอทิลีนไกลคอลเข้าสู่เซลล์คาโค-2 ในการศึกษาใช้แคลซีนและแคลซีนอะซีทอกซีเมทิลเอสเตอร์ (แคลซีนเอเอ็ม) เป็นตัวแทนของสารที่ละลายน้ำได้ดีและสารที่เป็นซับสเตรทของพี-ไกลโคโปรตีนซึ่งละลายในไขมันได้ดีตามลำดับ ลิโพโซมที่บรรจุแคลซีนและแคลซีนเอเอ็มที่ใช้ในการศึกษานี้ เตรียมขึ้นด้วยวิธีฟิล์มไฮเดรชันหรือดีไฮเดรชัน-รีไฮเดรชัน และนำมาลดขนาดจนมีขนาดประมาณ 100 นาโนเมตร ผู้วิจัยได้ศึกษาคุณสมบัติทางกายภาพของลิโพโซมและการนำส่งสารที่เป็นตัวแทนทั้งสองชนิดเข้าสู่เซลล์ โดยเปรียบเทียบระหว่างลิโพโซมที่มีส่วนประกอบแตกต่างกันและเทียบกับสารละลาย ผลการทดลองพบว่าลิโพโซมที่เตรียมจากฟอสโฟลิพิดแบบดั้งเดิมทุกชนิดที่ใช้ในการศึกษาสามารถเพิ่มการนำส่งแคลซีนเข้าสู่เซลล์คาโค-2 ได้ และการใส่พอลิเอทิลีนไกลคอลบนพื้นผิวลิโพโซมส่งผลให้การนำส่งแคลซีนเข้าสู่เซลล์ลดลงในทุกกรณี ในกลุ่มของลิโพโซมที่มีพอลิเอทิลีนไกลคอลพบว่ามีเพียงลิโพโซมที่มีประจุบวกเท่านั้นที่สามารถนำส่งแคลซีนเข้าสู่เซลล์ได้ดีกว่าสารละลาย สำหรับในกรณีของแคลซีนเอเอ็ม พบว่า ลิโพโซมแบบดั้งเดิมที่ไม่มีประจุและลิโพโซมชนิดที่มีพอลิเอทิลีนไกลคอลทั้งแบบไม่มีประจุและมีประจุบวกสามารถนำส่งสารเข้าสู่เซลล์ได้อย่างมีประสิทธิภาพ การใส่พอลิเอทิลีนไกลคอลบนพื้นผิวลิโพโซมชนิดไม่มีประจุและมีประจุลบไม่มีผลเปลี่ยนแปลงการนำส่งแคลซีนเอเอ็มเข้าสู่เซลล์เมื่อเทียบกับลิโพโซมชนิดที่ไม่มีพอลิเอทิลีนไกลคอล แต่การใส่พอลิเอทิลีนไกลคอลช่วยให้การนำส่งแคลซีนเอเอ็มของลิโพโซมประจุบวกเพิ่มขึ้นอย่างชัดเจน นอกจากนี้จากการใช้เทคนิคฟลูออเรสเซนดีเควนชิงชี้ว่า กลไกหลักที่ใช้นำส่งลิโพโซมที่มีพอลิเอทิลีนไกลคอลเข้าสู่เซลล์คือกระบวนการเอนโดไซโตซิสเช่นเดียวกับลิโพโซมแบบดั้งเดิม การศึกษานี้แสดงให้เห็นว่าส่วนประกอบของลิโพโซม อันได้แก่ ประจุบนพื้นผิวและการใส่พอลิเอทิลีนไกลคอล รวมถึงคุณสมบัติของสารที่นำมาบรรจุในลิโพโซม มีผลต่ออันตรกิริยาระหว่างลิโพโซมและเซลล์ ส่งผลให้ลิโพโซมเข้าสู่เซลล์ในปริมาณที่แตกต่างกัน ผลการศึกษาโดยรวมแสดงให้เห็นว่าการเลือกใส่ประจุที่เหมาะสมบนพื้นผิวของลิโพโซมชนิดที่มีพอลิเอทิลีนไกลคอลสามารถช่วยทำให้สารที่ละลายน้ำได้ดีและสารที่เป็นซับสเตรทของพี-ไกลโคโปรตีนเข้าสู่เซลล์ได้ดีขึ้น

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต .....

สาขาวิชา เทคโนโลยีเภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : PEGylation/ Liposomes/ P-glycoprotein/ Uptake/ Endocytosis/ Calcein/ Calcein AM/ Caco-2 cells

JITKASEM MEEWAN: EFFECT OF PEGYLATION ON THE DELIVERY OF PHOSPHOLIPID-BASED LIPOSOMES INTO CACO-2 CELLS. ADVISOR: ASST. PROF. NONTIMA VARDHANABHUTI, Ph.D., CO-ADVISOR: ASSOC. PROF. NUSARA PIYAPOLRUNGROJ, Ph.D., 95 pp.

This study was aimed to investigate the effects of PEGylation on cellular uptake of phospholipid-based liposomes into Caco-2 cells. The influence of liposome surface charge was also studied. The major mechanism in the delivery of PEGylated liposomes into Caco-2 cells was verified. Calcein and calcein acetoxymethyl ester (calcein AM) were used as models for hydrophilic compounds and lipophilic P-gp substrates, respectively. Calcein and calcein AM-loaded liposomes were prepared by either the film hydration or the dehydration-rehydration method, followed by size reduction to approximately 100 nm. The uptake of liposomes by Caco-2 cells was compared among liposomes with various compositions and with the solutions. The calcein uptake study showed that all types of conventional liposomes could enhance calcein uptake into Caco-2 cells. The calcein uptake from PEGylated liposomes was less efficient than that from conventional liposomes in all cases. Among the PEGylated liposomes, only those with positive surface charge showed better uptake efficiency than that of the calcein solution. For calcein AM, conventional neutral, PEG neutral, and PEG positively charged liposomes were successful in enhancing the uptake of the P-gp substrate by Caco-2 cells. PEGylation did not result in any changes in calcein AM uptake for both neutral and negatively charged liposomes. However, PEGylation markedly increased calcein AM uptake in positively charged liposomes. Furthermore, the use of fluorescence dequenching technique indicated that the major mechanism of PEGylated liposome uptake was likely to be endocytosis, similar to that of the conventional liposomes. This study indicated that liposome compositions including surface charge and PEGylation as well as the properties of the liposome contents would influence liposome-cell interaction, leading to the difference in liposome uptake. The overall results also indicated that PEGylated liposomes with the right composition could improve the delivery of hydrophilic compounds and lipophilic P-gp substrates into Caco-2 cells.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AEF	accumulation enhancement factor
Caco-2	human colon adenocarcinoma
calcein AM	calcein acetoxymetyl ester
CH	cholesterol
CV1	African green monkey kidney cell line
DCP	dicetylphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
EPR	enhanced permeability and retention
FBS	fetal bovine serum
HeLa	human ovarian carcinoma cell line
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
J774	murine derived mononuclear macrophage cell line
MDR	multidrug resistance
mg	milligram
mL	milliliter
mM	millimolar
MRP	multidrug resistance-associated protein
MRP2	multidrug resistance-associated protein 2
MW	molecular weight
nm	nanometer
nM	nanomolar
PEG2000-PE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
P-gp	P-glycoprotein
PBS	phosphate buffer saline
PEG	polyethylene glycol

RES	reticuloendothelial system
SA	stearylamine
SD	standard deviation
SEM	standard error of means
SPC	soybean phosphatidylcholne
µg	microgram
µl	microliter
µM	micromolar

CHAPTER I

INTRODUCTION

The most convenient and the most preferred route of drug administration is the oral route. However, low bioavailability of many drugs due to physical and biochemical barriers in the gastrointestinal (GI) tract are still of major concern for pharmaceutical scientists (Ensign, Cone and Hanes, 2012; Sant et al., 2012). Before reaching their final destinations, drugs administered orally at least have to go through the stomach into the lumen of the intestine and then across the intestinal epithelium. Hydrophilic drugs usually have limited GI absorption due to low membrane permeability. In addition, the lumen of the small intestine contains a thick mucus layer, bile salts, and pancreatic enzymes. These typical characteristics of the GI tract may lead to poor bioavailability of several drugs (Goldberg and Gomez-Orellana, 2003; Sant et al., 2012). The enzymatic and physical environment of the GI tract may affect the efficiency of the oral drugs. The drugs can be dramatically degraded by the highly acidic condition in the stomach and the digestive enzymes in the intestinal lumen before absorption (Allémann, Leroux and Gurny, 1998; Goldberg and Gomez-Orellana, 2003).

Another main type of absorption barrier in the GI tract is the efflux transporters. The efflux transporters are found on both the apical and the basolateral membranes of epithelial cells in the GI tract. The role of the efflux transporters is to prevent substrate accumulation in the cells by increasing substrate efflux out of the cells (Schinkel and Jonker, 2003; Takano, Yumoto, and Murakami, 2006). Those that are on the apical membranes are generally considered the biochemical barrier to drug absorption. The efflux transporters found in the GI tract include both the multidrug resistance and the multidrug resistance-associated proteins (Chan, Lowes and Hirst, 2004). The most widely studied efflux transporter in the GI tract to date is the P-glycoprotein (P-gp) (Bansal et al., 2009; Varma, Perumal and Panchagnula, 2006).

P-gp confers a protective role in the elimination of its substrates in a variety of normal human tissues including the liver, kidney, brain, adrenal gland, and intestinal epithelia (Fardel, Lecureur and Guillouzo, 1996; Loo and Clarke, 2005). In

the small intestine it is localized in the apical membrane (Washington, Washington, and Wilson, 2001). Many drugs are P-gp substrates. These include cardiac glycosides, immunosuppressive agents, calcium channel blockers and HIV protease inhibitors (Gavhane and Yadav, 2012; Linnet and Ejsing, 2008; Murthy and Shah, 2007). These P-gp substrates have shown relatively low oral bioavailability (Gavhane and Yadav, 2012; Guan et al., 2011; Westphal et al., 2000). Apart from normal tissues, overexpression of P-gp has been found in various cancer cells. P-gp prevents intracellular accumulation of many anticancer drugs that are its substrates and causes multidrug resistance (MDR) (Bansal et al., 2009; Krishna and Mayer, 2000; Nobili et al., 2012). The inhibition of the P-gp efflux pump can enhance intracellular accumulation of P-gp substrates, resulting in more effective treatments with these drugs (Papahadjopoulos et al., 1991; Zhao et al., 2007).

Various strategies have been used to inhibit or bypass P-gp function. The P-gp substrates thereby do not get effluxed out of the cells, resulting in more effective treatments (Bansal et al., 2009). The two main approaches are the use of P-gp modulators and the use of drug delivery systems (DDSs) (Bansal et al., 2009; Murthy and Shah, 2007). P-gp modulators mostly comprise pharmacologically active agents that are used for other indications such as verapamil, cyclosporine A, nelfinavir, quinidine, and valspoda (PSC833) (Nobili et al., 2012; Varma et al., 2003). These modulators can enhance intracellular accumulation of P-gp substrates. However, they have to be used at concentrations much higher than those required for their therapeutic activity, leading to toxicities (Krishna and Mayer, 2000). Scientific evidence indicates that DDSs such as liposomes, micelles and polymer-lipid hybrid nanoparticles can enhance intracellular drug accumulation, increase cellular uptake, and also decrease drug efflux by P-gp (Dabholkar et al., 2006; Ho et al., 2006; Michieli et al., 1999; Zastre, Jackson and Burt, 2004).

Liposomes have been extensively studied as colloidal drug carriers to maximize the efficacy of various therapeutic drugs and have become clinically accepted in cancer therapy. Both hydrophilic and hydrophobic drugs can be incorporated in liposomes. Liposome delivery systems have been principally used for parenteral administration. Liposomal drugs show relatively prolonged systemic circulation time and increased accumulation in solid tumors in comparison with the

drug in solution due to the enhanced permeability and retention effect (Drummond et al., 1999; Papahadjopoulos et al., 1991). However, one major drawback of liposomes is that conventional liposomes are still rapidly cleared from the systemic circulation by the reticuloendothelial system (RES). This is one of many limitations to the in vivo application of conventional liposomes as drug carriers (Papahadjopoulos et al., 1991).

Consequently, the development of long circulating liposomes introduced the concept of sterically stabilized liposomes. The first successful attempt was the creation of Doxil®, a PEGylated liposome-encapsulated form of doxorubicin. Doxil® shows a superior efficiency in cancer therapy with lower toxicity, compared to the free drug (Gabizon, 2001). Conjugation of polyethylene glycol (PEG) on the surface of liposomes can avoid rapid entrapment by the RES since the PEG chains form a hydrophilic, steric barrier on the liposome surface. The hydrophilic barrier stabilizes the complex and prevents opsonization and the subsequent interaction of liposomes with macrophages (des Rieux et al., 2006; Torchilin and Trubetskoy, 1995). These lead to prolonged circulation time of liposomes when administered parenterally.

Attempts to use liposomes as oral drug delivery systems have been a major interest in the past 30 years. The properties of liposomes in protecting the entrapped drugs from digestive degradation or increasing the absorption of poorly absorbed drugs from the GI tract were raised (Chiang and Weiner, 1987). However, a major obstacle in using conventional liposomes as an oral dosage form is the instability of the liposomes themselves in the GI tract. Conventional liposomes are susceptible to rapid degradation by the acids, the bile salts and the digestive enzymes in the GI tract (Ariën et al., 1993; Tobío et al., 2000). In order to improve their stability and thus oral bioavailability, various polymers have been used as coating materials (Chen, Torchilin, and Langer, 1996; Tobío et al., 2000). Conjugation with PEG has been shown not only to prevent opsonization and the subsequent uptake by macrophages but also to increase enzymatic stability, resulting in much more stable liposome vesicles upon contact with the digestive fluids (Iwanaga et al., 1997; Tobío et al., 2000). Therefore, sterically stabilized liposomes by PEG should also be promising as a delivery system for the oral route.

There are some studies associated with using liposome technology to bypass P-gp in intestinal epithelial cells (Lo, Liu and Cherng, 2001; Lo, 2000; Mamot et al.,

2003). Liposome formulations can increase the absorption and decrease the efflux of epirubicin, a P-gp substrate, across human intestinal epithelial Caco-2 monolayers (Lo, 2000). In addition, neutral and negatively charged liposomes have been reported to enhance uptake of the P-gp substrate calcein AM into Caco-2 cells by endocytosis (Ing-orn Prasanchaimontri, 2009).

Both liposome composition and type of cells have been shown to influence the liposome-cell interaction (Lee, Hong and Papahadjopoulos, 1992; Miller et al., 1998). Although the presence of PEG on liposome surface has been reported to reduce endocytosis when compared to conventional liposomes in some specific cell lines, they can still be able to deliver their contents efficiently to those cells (Miller et al., 1998). Thus, PEGylated liposomes may be useful to enhance the delivery of P-gp substrates to intestinal epithelial cells or to other cells that express P-gp. However, the role of PEGylated liposomes in drug delivery to the human intestinal epithelial cells in terms of both uptake efficiency and the liposome-cell interaction has not been reported. Furthermore, it has been suggested that incorporating surface charges can increase entrapment efficiency of ionizable compounds and decrease aggregation of liposomes (Lasic and Martin, 1995). None of the effects of surface charges have been studied on the PEGylated liposomes or on their uptake into the human intestinal epithelial cells.

In this study, the effects of PEGylation on the uptake of liposomes into the human intestinal epithelial cells were studied to evaluate the plausibility of its application for the oral route. The effects of inclusion of charged lipids in PEGylated liposomes on the cellular liposome uptake were also investigated. Furthermore, the feasibility of using PEGylated liposomes to enhance the delivery of lowly permeable drugs, either hydrophilic drugs or lipophilic P-gp substrates, was evaluated in comparison with conventional liposomes. The uptake efficiency of PEGylated liposomes with and without charge was compared with that of the conventional liposomes. Moreover, the major mechanism in the uptake of PEGylated liposomes into the intestinal epithelial cells was verified.

Caco-2 cells, which were propagated from a well-established epithelial human colon adenocarcinoma cell line, were used as a model for intestinal epithelial cells in this study. This cell line is well accepted for the studies of drug transport in

the GI tract as well as the studies of P-gp efflux transporters (Artursson and Borchardt, 1997; Balimane and Chong, 2005; Hunter et al., 1993). Calcein, a fluorescent dye, was used as a model of hydrophilic compounds entrapped in liposomes. This compound also served as the aqueous phase marker for liposomes. Calcein AM was used as a model compound for lipophilic P-gp substrates (Chan, Lowes, and Hirst, 2004; Eneroth et al., 2001). These two compounds are widely used in liposome research (Chu et al., 1990; Connor and Huang, 1985; Ing-orn Prasanchaimontri, 2009). The findings obtained from this study will be useful information for further development of sterically stabilized liposomes as oral delivery systems and also, to some extent, to overcome MDR in colon cancers.

Objectives

The overall objective of this study was to evaluate the plausibility of using PEGylated liposomes for oral drug delivery. The specific objectives of this study were as follows:

1. To compare the uptake efficiency of PEGylated liposomes with that of conventional phospholipid-based liposomes in Caco-2 cells
2. To evaluate the effect of inclusion of surface charge into PEGylated liposomes on liposome uptake by Caco-2 cells
3. To evaluate the plausibility of using PEGylated liposomes to enhance the delivery of hydrophilic compounds and to bypass the function of P-gp in Caco-2 cells
4. To verify the major mechanism in the uptake of PEGylated liposomes by Caco-2 cells

CHAPTER II

LITERATURE REVIEW

Oral delivery is the most employed route and the most convenient way of drug administration. However, many drugs are not suitable to be administered via the oral route due to several factors, resulting in low oral bioavailability.

Barriers for oral drug delivery

The unique physiological conditions in the GI tract that lead to low oral bioavailability of several drugs are summarized as follows.

1. Gastric pH and digestive enzymes (Ensign, Cone and Hanes, 2012; Washington, Washington and Wilson, 2001)

Drug molecules need to be resistant to the harsh environment in the GI tract. The pH in the GI tract is not uniform, ranging from 1 to 2 in the stomach, then steadily increases in the duodenum and reaching a pH of 7–8 in the colon and rectum. GI tract also contains several digestive enzymes. For instance, the lumen of the small intestine contains various pancreatic enzymes including pancreatic proteases, amylases, and lipases. Bile salts which are secreted by liver are powerful natural surfactants associated with the digestion and the absorption of lipids. Bile salt concentration has been reported to be around 10.8 mM in human duodenum (Richards and Gardner, 1978). Many drugs especially compounds classified in BCS class III, which are high solubility but low permeability, are generally susceptible to the degradation in the GI tract before absorption through the epithelial cells of small intestine (Sant et al., 2012).

2. Mucus layers (Ensign, Cone and Hanes, 2012; Washington, Washington and Wilson, 2001)

The mucus layers which protect epithelial surfaces have been focused as another important barrier for oral drug absorption. Mucus has a variety of functions. For instance, it restricts the penetration of large molecules, lubricates the materials passing through the GI tract, and prevents invasion of pathogenic bacteria. The most important property of mucus is viscoelasticity, which are essential for its lubricating and protective properties. The thickness of the mucus layer varies throughout the GI

tract. Mucus is thickest in the stomach (40–450 μm) where it serves to protect the stomach from being digested by gastric acid and proteolytic enzymes. In the small intestine, types of food influence the thickness of mucus barrier. The transport of large compounds can be restricted by this layer as a result of low mucosal permeability. Mucoadhesives and permeation enhancers have been introduced to disrupt barrier properties of mucus lining to improve delivery in the GI tract. Furthermore, the intestinal epithelium which is covered by mucus offers poor permeability for most water soluble molecules.

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

Efflux transporters are proteins expressed on the cell membranes. The efflux transporters in the GI tract include both the multidrug resistance (MDR) and the multidrug resistance-associated proteins (MRPs). The function of efflux transporters is to increase substrate efflux out of the cells resulting in limited cellular accumulation of the substrates. This efflux function results in low bioavailability of the substrates. Among various types of efflux transporter, P-glycoprotein (P-gp) is one of the most extensively investigated.

P-glycoprotein (Chan, Lowes and Hirst, 2004; Fardel, Lecureur and Guillouzo, 1996; Krishna and Mayer, 2000; Varma et al., 2003)

The function of P-gp is to prevent intracellular accumulation of its substrates in an ATP-dependent manner. The expression of P-gp has been found in various normal tissues such as epithelial cells of kidneys, liver, small intestine, colon, pancreas, placenta and endothelial cells at blood-tissue barriers such as the blood-brain barrier. In addition, the overexpression of P-gp has been found in both solid tumors and hematological malignancies. In cancer cells, P-gp causes multidrug resistance to anticancer drugs.

P-gp substrates

The substrates of P-gp include mostly a number of structural and functional unrelated hydrophobic compounds. Many groups of drug have been shown to be P-gp substrates such as anticancer agents, cardiac glycosides, immunosuppressive agents, calcium channel blockers, HIV protease inhibitors. Examples of P-gp substrates are shown in Table 1.

Table 1: Examples of P-gp substrates (Chan et al., 2004; Gavhane and Yadav, 2012; Linnet and Ejsing, 2008; Murthy and Shah, 2007; Varma et al., 2003)

Substrate class	Examples
Anticancer agents	Vinca alkaloids (Vincristine, Vinblastine)
	Taxanes (Paclitaxel)
	Anthracyclins (Doxorubicin, Daunorubicin)
	Epipodophyllotoxins (Teniposode, Etoposide)
Immunosuppressants	Cyclosporin A, Tacrolimus
Cardiac glycosides	Digoxin, Quinidine
Glucocorticoids	Dexamethasone, Methylprednisolone
Anthelmintics	Ivermectine
HIV protease inhibitors	Saquinavir, Indinavir, Ritonavir
Ca^{++} channel blockers	Verapamil, Diltiazem, Nifedipine, Felodipine
Fluorescent dyes	Calcein-AM, Fluro-2, Rhodamine 12

Approaches to overcome the function of P-gp

Various strategies have been used to bypass the function of P-gp. Two prevalent approaches in order to enhance the delivery of P-gp substrates are the use of P-gp modulators/inhibitors and the use of drug delivery systems (Bansal et al., 2009).

1. The use of P-gp modulators/inhibitors (Green, Marder and Slapak, 2001; Krishna and Mayer, 2000; Murthy and Shah, 2007; Nobili et al., 2012; Varma et al., 2003; Wandel et al., 1999)

P-gp modulators were developed to modulate P-gp function. They are classified into three generations based on their specificity and affinity. Many of P-gp modulators are themselves found to be substrates of P-gp and thus inhibit the P-gp efflux by competing with other P-gp substrates. Compounds that have been identified as P-gp inhibitors are listed in Table 2.

Table 2: List of P-gp modulators/inhibitors (Murthy and Shah, 2007)

Generation of P-gp modulators	Compounds classified
1st generation	Verapamil, Nifedipine, Cyclosporin A, Tamoxifen, Trifluoperazine, Cremophor EL, Progesterone, Quinidine, Dipyridamole
2nd generation	Valspodar (PSC 833), Biricodar (VX 710), Dexverapamil, Dexniguldipine, Rapamycin
3rd generation	Zosuquidar (LY335979), Laniquidar (R10193), Tariquidar (XR9576), Substituted diarylimidazole (ONT093)

First generation P-gp modulators

First generation P-gp modulators are pharmacologically active agents that are used for treatment of pathological conditions. These include immunosuppressants such as cyclosporine A and calcium channel blockers such as verapamil. First generation P-gp modulators demonstrate low binding affinity to the P-gp efflux pump. Though these modulators can be used to improve intracellular accumulation of P-gp substrates, they have to be used at higher concentrations than those required for their therapeutic activity leading to toxicities. Therefore, these compounds have no practical use as P-gp modulators in clinical practice (Nobili et al., 2012).

Second generation P-gp modulators

Second generation P-gp modulators have been developed to decrease side effects associated with the first generation compounds. They are less toxic and much more potent than the first generation P-gp modulators. Moreover, they show the lack of pharmacological activity and a higher P-gp affinity.

Both first and the second generation modulators are themselves P-gp substrates and compete as a substrate for binding to the pump. However, these first and second generation P-gp modulators can be substrates or inhibitors of CYP450-3A and can be substrates of other ABC transporters. This can lead to a possibility of complicated drug-drug interactions as well as pharmacokinetic alterations.

Third generation P-gp modulators

Third generation P-gp modulators have high affinity towards P-gp and therefore can be used in the nanomolar range. This type of modulator binds to the pump with high affinity but is not itself substrate. This induces a protein conformational change which prevents ATP hydrolysis and the transportation of the substrates out of the cells, resulting in an increased intracellular accumulation. Furthermore, it was observed that these agents did not affect CYP450-3A4 (Nobili et al., 2012). Therefore, they are not likely to alter the pharmacokinetics of drugs that are P-gp substrates. However, compounds in this generation are still under development.

2. The use of drug delivery systems

The use of drug delivery systems has become an interesting alternative to the use of P-gp modulators. Several drug delivery systems such as micelles, nanostructured lipid carriers, polymer-lipid hybrid nanoparticles, and liposomes have been used to overcome the function of P-gp efflux transporters (Beloqui et al., 2013; Dabholkar et al., 2006; Ho et al., 2006; Michieli et al., 1999; Zastre, Jackson and Burt, 2004).

Micelles have been used successfully to bypass P-gp function. The study of Dabholkar et al. (2006) has revealed that mixed micelles could efficiently bypass P-gp function of P-gp substrates, paclitaxel and rhodamine-123 in Caco-2 cells. Besides micelles, other drug carriers have also been shown to be potential tools for the delivery of P-gp substrates. Di-block copolymers have been used successfully to reduce doxorubicin and rhodamine-123 efflux through the inhibition of P-gp function in various types of cancer cells (Elamanchili, McEachern and Burt, 2009; Zastre et al., 2004). It was reported that nanostructured lipid carriers showed a success improvement in the oral bioavailability of a poorly water-soluble P-gp substrate, saquinavir, across the intestinal barrier (Beloqui et al., 2013). A polymer-lipid hybrid nanoparticulate system has also shown its potential to enhance cellular accumulation and retention of doxorubicin in the P-gp-overexpressing tumor cells (Ho et al., 2006).

Liposomes have been extensively studied as colloidal drug carriers to maximize the efficacy of various therapeutic drugs including P-gp substrates (Murthy and Shah, 2007). Both hydrophilic and hydrophobic drugs can be incorporated in

liposomes. Liposomal carriers have been used successfully to bypass the function of P-gp. Most of P-gp substrates studied with liposomes are anticancer drugs such as daunorubicin (Michieli et al., 1999; Pratt et al., 1998) and doxorubicin (Coukell and Spencer, 1997; Riganti et al., 2011; Sugiyama and Sadzuka, 2013). Furthermore, liposome technology can be used to bypass P-gp in GI epithelial cells (Kobayashi et al., 2007; Lo, Liu, and Cherng, 2001; Mamot et al., 2003). Liposome formulations showed an increased absorption and a reduced efflux of the P-gp substrate epirubicin across human intestinal epithelial Caco-2 monolayers (Lo, 2000). Another study in which liposomes carrying neutral and negatively charges were successful in enhancing the uptake of the P-gp substrate calcein AM into Caco-2 cells by endocytosis was reported (Ing-orn Prasanchaimontri, 2009).

Liposomes as drug carriers

Though some liposome products have become clinically accepted and approved by US FDA for the use in cancer treatment, they have been principally designed for parenteral administration in order to protect labile active molecules against undesirable environment and to reach specific sites. Liposomal drugs show relatively prolonged circulation time and increased accumulation in solid tumors compared to drugs in solution due to the enhanced permeability and retention (EPR) effect (Drummond et al., 1999; Papahadjopoulos et al., 1991).

EPR effect is caused by the leakiness of tumor microvessels, which allows liposomes, small particles, and macromolecules entry into the tumor interstitium, as well as the lack of a functional lymphatic drainage in tumors. These create a “dead-end” for extravasated liposomes (Gabizon, 2001). However, one of many limitations to the in vivo application of liposomes as drug carriers is that liposomes are rapidly cleared from systemic circulation by the reticuloendothelial system (RES) (Papahadjopoulos et al., 1991). Consequently, the development of long circulating liposomes has introduced the concept of sterically stabilized liposomes into liposome technology.

PEG-coated liposomes

The steric stabilization is introduced to protect the surface of vesicles by grafting the polymer chain onto the liposome surface (Torchilin and Trubetskoy, 1995). One of the most successful and popular methods to obtain long circulating stable liposomes is their coating with flexible, amphipathic polymers, especially polyethylene glycol (PEG). Some hydrophobic derivatives of PEG (usually PEG-PE) are commercially available. The first successful attempt was the creation of Doxil®, a PEGylated liposome-encapsulated form of doxorubicin. Doxil® shows a superior efficiency in cancer therapy and lower toxicity, compared to the free drug (Gabizon, 2001). Long circulation of PEG-coated liposomes can be explained by the role of surface charge and hydrophilicity of PEGylated liposomes, the role of PEG in the repulsive interactions between PEG on the liposome membrane and another liposome or cell, the formation of a dense 'conformational cloud' by flexible polymers over the liposome surface, and the decreased rate of plasma protein adsorption (Torchilin and Weissig, 2003). PEG on the liposome surface can escape recognition by cells as a result of a protective layer of the PEG over the liposome surface. The hydrophilic barrier stabilizes the complex and prevents opsonization and the subsequent interactions of liposomes with macrophages resulting in avoid rapid entrapment by the RES. These lead to prolonged liposome circulation when administered parenterally (des Rieux et al., 2006; Romberg, Hennink and Storm, 2008; Torchilin and Trubetskoy, 1995).

The steric stabilization is affected by three major factors including liposome size, PEG chain length, and PEG domain density (Klibanov et al., 1991; Mori et al., 1991; Singh, Singh and Lillard, 2008).

1. Liposome size

Relatively small liposomes with the size smaller than 200 nm are long-circulating in nature. Liposome size larger than 300 nm leads to the increased tendency to accumulate in spleen. Very small liposomes with average diameter less than 70 nm have shown an increased accumulation in liver (Klibanov et al., 1991; Litzinger et al., 1994; Torchilin and Weissig, 2003). These liposomes can penetrate through the fenestrae of the liver endothelium and become localized in the parenchymal cells of the liver. The ability of PEG in reducing protein binding

decreased significantly for the very small liposomes. This can be explained by an increase in the curvature of the liposomes. As the curvature increases, the extended chains spread to a greater degree, which refers to an increased space between bristles of a hair brush. Proteins including opsonins can thereby easily penetrate between polymer chains of the very small liposomes (Litzinger et al., 1994). These findings suggest that the size of liposomes used for drug delivery should be chosen carefully. The size range that PEGylation can offer its best benefit should be 100-200 nm. This proper size range should be applied to the case of potential interaction between PEGylated liposomes with other endogenous protein molecules such as enzymes in the GI tract as well.

2. PEG chain length

PEG chain length also affects liposome circulation time and steric barrier activity. The steric barrier property increases as the PEG chain length increases. However, the steric barrier activity of PEG5000-PE is too strong, causing a reduced target binding of immunoliposomes. Therefore, PEG2000-PE seems to be the optimal length of PEG (Mori et al., 1991).

3. PEG domain density

The carefully chosen PEG concentration for the steric protection effects should be concerned. A relatively low concentration of the flexible PEG molecules (approximately 1 %) is sufficient to create high density conformational clouds over the liposome surface, resulting in protecting the liposomes from opsonization (Torchilin et al., 1994). However, it was detected that penetrating molecules could still diffuse through the liposome surface at low PEG concentrations. This diffusion can be blocked almost completely by increasing PEG concentration. Another experiment indicated that low levels of PEG polymer on the liposome surface (< 2%) did not significantly prevent the attack by protein molecules but hinder close contact with another liposome (Klibanov et al., 1991). However, at higher concentrations of PEG, these events are blocked. The residence time of liposomes in the blood was studied with the incorporation of PEG in the range of 2.5 to 6.5 mol%. The clearance rate was faster in the liposomes with low PEG concentration. Furthermore, it was also reported that 7 mol% of PEG-PE provided maximum steric protection due to complete coating over the liposome surface (Torchilin and Trubetskoy, 1995). To

prepare PEGylated liposomes, the required quantity of PEG is usually 2-8 mol% of total lipids. However, PEG at 5 mol% is generally used in preparing PEGylated liposomes in several studies and also in Doxil®, the product that has been approved for clinical applications (Miller et al., 1998; Papahadjopoulos et al., 1991; Torchilin and Weissig, 2003; Zhao et al., 2007).

Liposomes as oral drug carriers

The most convenient and the most often-used means of drug administration is via the oral route. Thus, attempts have been made toward liposomal delivery systems. Liposomes have mostly been studied for the oral route to improve the oral bioavailability of BCS class III drugs such as proteins by protecting them from the degradation in the GI tract and improving the intestinal absorption (Singh et al., 2008; Werle and Takeuchi, 2009). Unfortunately, the major obstacle is that most conventional liposomes cannot be used for oral delivery due to their instability in the GI tract. They are not stable at the acidic pH in the stomach. Furthermore, the structural phospholipids are susceptible to hydrolysis by pancreatic lipases and micelle formation with bile salts can demolish the vesicular structure of phospholipid-based liposomes. Bile salts are water-soluble biological detergents which can create micelles as depicted in Figure 1. Disruption of the liposome membrane leads to the exposure of encapsulated substances and thereby the loss of their protective functions (Arien et al., 1993; Chiang and Weiner, 1987; Tobío et al., 2000).

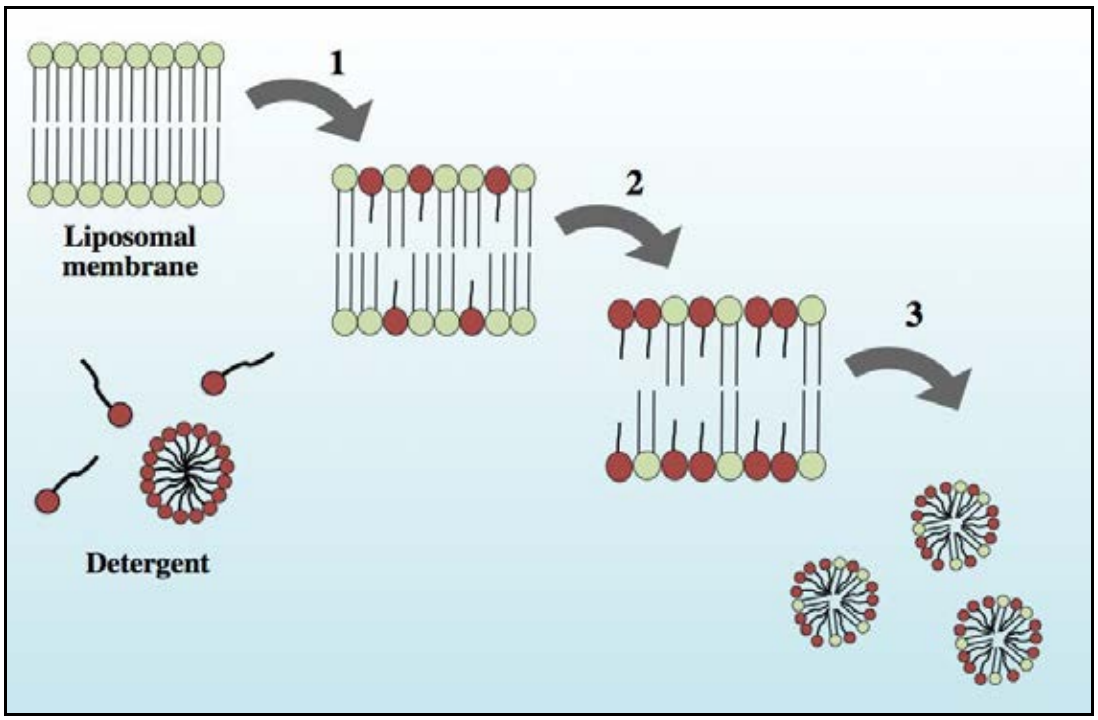

Figure 1: A schematic of liposome solubilization dynamics. 1. Lipid membrane of liposomes is attacked by surfactant molecules 2. Amount of surfactant molecules gradually increases in a liposome membrane and the liposome size becomes larger 3. Mixed micelles are released from the liposome and the mother liposome finally collapses (modified from Shoji et al., 2012)

Liposomes composed of lipids with high transition temperatures, such as dipalmitoyl-phosphatidylcholine (DPPC) and distearoyl-phosphatidylcholine (DSPC), demonstrate longer membrane integrity retention than liposomes composed of lipids with low transition temperatures (phosphatidylcholine (PC)) in the presence of bile salts, especially when cholesterol is added (Kokkona et al., 2000; Richards and Gardner, 1978). This is possibly caused by the difficulty for the bile salts to be incorporated into the bilayers and to perturb the structure of the liposomes having high phase-transition temperature.

In order to improve liposome oral bioavailability, various polymers are used as coating materials. Examples of polymers that have been studied for potential oral liposomal drug delivery include chitosan, poly (lactide-co-glycolide) (PLG), poly(alkyl cyanoacrylate), and PEG (Allémann, Leroux and Gurny, 1998; Chen and

Langer, 1998; Chen, Torchilin and Langer, 1996; Singh et al., 2008; Swaminathan and Ehrhardt, 2012; Tobío et al., 2000). Among these, PEG is the most extensively explored. PEG is considered to be a nontoxic hydrophilic polymer with FDA approval and has been used in pharmaceutical and medical applications for decades (Singh et al., 2008).

The advantages of PEGylation on nanoparticle stability have been well established for the oral route. PEG can increase particle stability in gastric and intestinal fluids, and against enzymatic attack especially the lipases (Tobío et al., 2000). The results from an in vitro study suggested that coating PEG over the liposome surface could create much more stable liposome vesicles upon contact with the digestive fluids (Iwanaga et al., 1999). Another interesting advantage of PEG for oral delivery is its mucoadhesion with the penetration of PEG chains into the mucous (Peppas, 1998).

Interactions of liposomes with cells (New, 1989; Torchilin and Weissig, 2003)

Liposomes can interact with cells in several ways to allow liposomal components to become associated with those cells. Figure 2 depicts possible interactions between liposomes and cells.

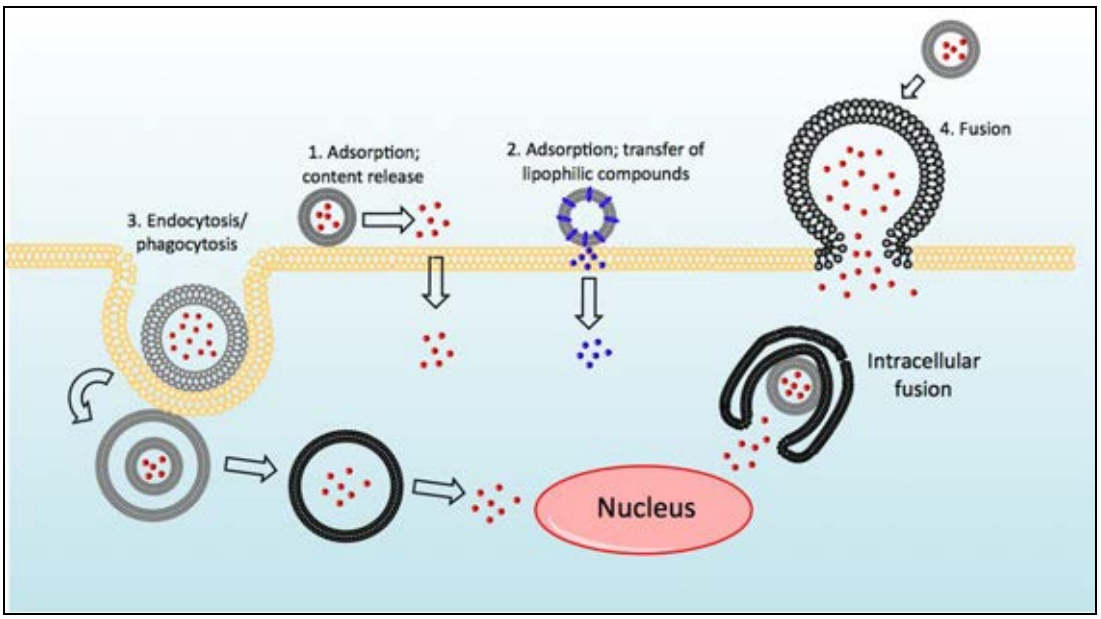

Figure 2: Possible interactions between liposomes and cells. 1. Adsorption of the liposome followed by the release of contents entrapped in the liposome 2. Adsorption of the liposome and subsequent transfer of lipophilic substances from the bilayer of the liposome to the bilayer of the cell 3. Endocytosis/phagocytosis of the liposome followed by intracellular degradation of the liposome by the endolysosomal pathway 4. Fusion of the liposome membrane with the cell membrane and subsequent release the liposome contents in the cytoplasm (modified from Torchilin and Weissig, 2003)

The description of each mechanism is as follows.

1. Adsorption

Adsorption of liposomes to the cell surface may occur with little or no internalization of either aqueous or lipid components. In some cases, liposomes may remain adsorbed on the cell surface with complete retention of aqueous and lipid contents within the liposomes. In other cases, initial adsorption may result in subsequent interactions such as contact release or fusion.

2. Contact release

Liposome contact with the cells may cause an increase in permeability of the liposome membrane. This results in a release of solute molecules entrapped in the aqueous compartment of liposomes directly into the cells.

3. Intermembrane transfer

Intermembrane transfer of lipid components can take place upon close contact of the two phospholipid bilayers without disruption of liposome integrity. Lipophilic compounds in the liposome membrane can insert themselves into the cell membrane. Such transfer may occur with complete retention of the components in the aqueous compartment of liposomes.

4. Fusion

Close approach of liposome and cell membrane can lead to fusion. Fusion of liposomes with cells occurs by complete mixing of the liposome membrane with the cell membrane and releasing contents entrapped in the liposome to the cytoplasm.

5. Endocytosis

Liposomes bind to the cell surface and then are endocytosed by invagination of the plasma membrane into endosomes, which have a pH of 5 to 5.5. These endosomes then fuse with lysosomes to form secondary lysosomes where lysosomal digestion occurs at approximately pH 4.5. Lysosomal enzymes break the liposomes. The liposomal phospholipids are hydrolyzed to fatty acids, which can be either released out of the cells or be recycled and reincorporated into host phospholipids. During liposomes breakdown in lysosomes, the liposomal contents are released. These contents will either remain sequestered in the lysosomes until exocytosis or they will slowly leak out of the lysosome into the cell, depending on their physicochemical properties.

Most of liposomes are reported to be taken up into the cells by endocytosis (Medina-Kauwe, Xie and Hamm-Alvarez, 2005; Ziello, Huang and Jovin, 2010). However, the physicochemical properties and surface modification of liposomes may influence intracellular uptake mechanism of liposomes (Un et al., 2012). Neutral phosphatidylcholine liposomes are endocytosed by the cells, whereas negatively charged liposomes consisting of phosphatidylserine and phosphatidylcholine are taken up into the cells by fusion (Papahadjopoulos, Poste and Schaeffer, 1973; Poste and Papahadjopoulos, 1976).

Interactions of PEGylated liposomes with cells

Liposome-cell interaction has been influenced by both liposome composition and type of cells (Chan et al., 2012; Lee, Hong and Papahadjopoulos, 1992; Miller et al., 1998). Effect of PEGylation on the liposome uptake has also been investigated in several studies (Chan et al., 2012; Jung et al., 2009; Miller et al., 1998; Wang et al., 2010). The effect of liposome surface charge on both conventional and PEGylated liposomes was studied in two different cell lines: a human ovarian carcinoma cell line (HeLa) and a murine derived mononuclear macrophage cell line (J774) (Miller et al., 1998). The uptake of liposomes with similar compositions by these two cell types was different. In HeLa cells, the positively charged liposomes resulted in higher endocytosis than the neutral and the negatively charged liposomes. On the contrary, the greater interaction of liposomes with J774 cells was observed in both types of the charged liposomes than in the uncharged liposomes. However, PEGylated liposomes was obviously less endocytosed than conventional liposomes. The transfection efficiency of PEGylated cationic liposomes-DNA complexes in mouse fibroblast L-cells was reported to be less than those without PEG. These complexes were also reported to be taken up by the cells via endocytosis (Chan et al., 2012). Furthermore, PEG-complexed cationic liposomes demonstrated superior intracellular uptake over Doxil®, the PEGylated phospholipid-based liposomes, in B16F10 cells (Jung et al., 2009). However, the interaction of PEGylated liposomes and the human intestinal epithelial cells in terms of both uptake efficiency and the mechanism of cellular uptake has not been reported.

Use of markers to distinguish the difference between fusion and endocytosis

As mentioned earlier that most of liposomes are taken up into the cells by endocytosis. However, some liposome formulations especially those carrying negative charge are taken up into the cells by fusion. Thus, to distinguish the liposome-cell interaction between fusion and endocytosis is necessary.

Fluorescent markers can be used to discriminate the different liposome-cell interactions. In adsorption, the fluorescence is observed around the cell membrane (Manconi et al., 2007). Fusion and endocytosis show different fluorescent patterns in the cells. When both the labeled lipid and the aqueous marker are used, fusion shows

the fluorescence of lipid phase marker on the cell membrane, while the fluorescence of the aqueous phase marker diffuses in the cytoplasm. On the contrary, punctate fluorescence is observed in the endosomes or lysosomes if the interaction of liposomes with cells is mainly endocytosis as shown in Figure 3 (Chu et al., 1990; Connor and Huang, 1985). Moreover, double-labelled liposomes with different fluorescent colors will show co-localization of the two fluorescent markers within the cells (Lee et al., 1992; Manconi et al., 2007).

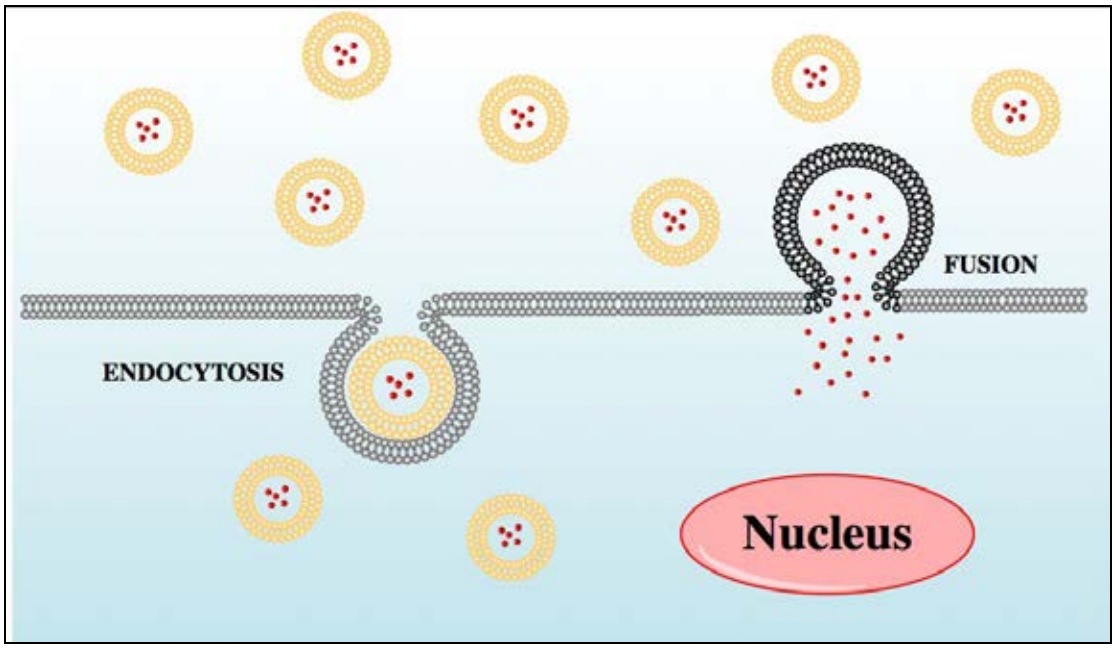

Figure 3: Two patterns of cellular uptake of liposomal calcein. In fusion, calcein, which is self-quenched when contained at high concentrations inside the liposome, shows a marked increase in the fluorescence since it spreads throughout the cytoplasm and become unquenched upon dilution. In contrast, uptake of liposomes by endocytosis causes no marked fluorescence increase since dilution of calcein contents does not occur. Most calcein is retained inside endosomes or lysosomes. (modified from New, 1989)

Another property of fluorescent markers that can be used to distinguish between fusion and endocytosis is self-quenching (New, 1989). The classic fluorescence dye used as an aqueous phase marker for liposomes is fluorescein

derivatives including carboxyfluorescein and calcein. At high concentration, carboxyfluorescein displays very little fluorescence, being self-quenched. Until the solution is diluted, the fluorescence consequently increases. Calcein which is less pH sensitive can be used instead of carboxyfluorescein. In fusion, calcein at a self-quenching concentration displays the strong fluorescence due to many hundred-fold dilutions in the cytoplasm. In endocytosis, in contrast, the dye shows very low fluorescence intensity when detected by the flow cytometric method as a result of the concentrated dye in endosomes or lysosomes. However, after cell lysis with detergents, the dilution of fluorescent calcein results in an increase in fluorescence intensity. Adsorbed liposomes will not display fluorescence at all, unless the free dye is leaked out.

Caco-2 as in vitro models (Artursson, Palm and Luthman, 2012; Natoli et al., 2011; Sambuy et al., 2005; Yu, Cook and Sinko, 1997)

The human intestinal Caco-2 cells have been extensively used as a model of the intestinal epithelial barrier. Caco-2 cells were obtained from a human colon adenocarcinoma. After spontaneous differentiation in long-term culture that leads to the formation of cell monolayers, they display several morphological and functional characteristics similar to the intestinal absorptive enterocytes. Caco-2 monolayers can be used as a model for theoretical predictions of drug absorption. Caco-2 cells have been also used to evaluate the cytotoxicity, drug–drug interaction, and metabolic stability. Similar to other carcinoma cell lines, several efflux pumps such as P-gp, MRP, breast cancer resistant protein (BCRP), and lung cancer-associated resistance protein are observed in Caco-2 cells when culture in an appropriate condition. Researchers have used Caco-2 cells to investigate the effect of P-gp on the uptake or transport of P-gp substrates such as vinblastine (Hunter, Hirst and Simmons, 1993), ritonavir (Holmstock, Annaert and Augustijns, 2012), and saquinavir (Beloqui et al., 2013). To evaluate the absorption and the permeability of various compounds, Caco-2 cells have also been used as a model of the intestinal barrier (Anderson et al., 2001; Beloqui et al., 2013; Iseki et al., 1997; Sambuy et al., 2005). Therefore, Caco-2 cells are suitable as an in vitro model cell culture for the study of drug transport in the GI tract and the effect of P-gp on the uptake of P-gp substrates.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Ammonium molybdate (Mallinckrodt, USA, Lot no. 3420X12465)
2. Calcein (Sigma, Japan, Lot no. 1461150)
3. Calcein acetoxymetyl ester (Fluka, USA, Lot no.41810P01)
4. Chloroform AR grade (Labscan, Ireland, Lot no. 09041093)
5. Cholesterol (Fluka, Japan, Lot no. 1324049)
6. Cyclosporin A (Sigma, China, Lot no. BCBD2418V)
7. Dicetylphosphate (Sigma, USA, Lot no. 10K1593)
8. Dimethyl sulphoxide (DMSO) (Sigma, USA, Lot no. 076K2321)
9. Disodium hydrogen orthophosphate (Univar, Australia, Lot no. F0J067)
10. Dulbecco's Modified Eagle's Medium (Gibco, USA, Lot nos. 984295, 1038180, 1165389))
11. Fetal bovine serum (Biochrom AG, Germany, Lot nos. 0859W, 0247X, 0317A)
12. Fiske- Subbarow reducer (Fluka, USA, Lot no. BCBF0620)
13. HEPES (N-[2-hydroxyethyl]piperazine-N'-[2- ethanesulfonic acid]) (Sigma, USA, Lot no. 040M5424)
14. Hydrogen peroxide solution (30% w/v) laboratory reagent grade (Merck, Germany, Lot no. K40578287)
15. Indomethacin (Sigma, USA, Lot no. 117F-0595)
16. L-glutamine (Gibco, Brazil, Lot nos. 946860, 1074912)
17. MEM non-essential amino acid (Gibco, USA, Lot no. 1150761)
18. MTT (Thiazolyl Blue Tetrazolium Bromide) (Sigma, USA, Lot no. 17296LH)
19. PEG2000-PE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) (Avanti Polar Lipid, USA, Lot no. 180PEG2PE-98)

20. Penicillin-Streptomycin (Gibco, USA, Lot nos. 1116257, 1141212)
21. Potassium dihydrogen phosphate (Merck, Germany, Lot no. A531473)
22. Sephadex G-50 (Sigma, Sweden, Lot no. 021M0124V)
23. Sodium bicarbonate (Carlo Erba, Italy, Lot no. Z2G569173M)
24. Sodium chloride (Merck, Germany, Lot no. K38447104)
25. Sodium hydroxide (Merck, Germany, Lot no. B0119798)
26. Soybean phosphatidylcholine (Epikuron 200) (Cargill, Germany, Lot no. 199060)
27. Stearylamine (Sigma, USA, Lot no. 45H3435)
28. Triton X-100 (Sigma, USA, Lot no. 40F1535)
29. Trypan blue (Sigma, USA, Lot no. 87F50385)
30. 0.05% Trypsin-EDTA (Gibco, Canada, Lot no. 939422)
31. 0.25% Trysin-EDTA (Gibco, Canada, Lot nos. 1176327, 1184654)
32. α-Tocopherol (Sigma, USA, Lot no. 063K0796)

Equipment

1. Analytical balances (AX/MX/UMX and AG285, Mettler Toledo, Switzerland)
2. Autoclave (Hirayama, Japan)
3. Centrifuge (Universal 320R, Hettich, Germany)
4. Counting chamber (BOECO, Germany)
5. CO_2 incubator (Forma series II water jacket, Thermo scientific, USA)
6. Electronic balance (BSA423S-CW, Sartorius Basic, Scientific Promation, co., Ltd., Thailand)
7. Flow cytometer (FACSCalibur, Becton Dickinson, USA)
8. Flex-column (Kontes, USA)
9. Hand-held extruder (LiposoFast™, AVESTIN, Canada)
10. Laminar hood biosafety cabinet class II (Microflow, Bioquell, UK)
11. Light microscope (CKX41, Olympus, Japan; IX51, Olympus, Japan)
12. Manifold freeze-dryer (Dura-Dry™ MP, USA)
13. Microplate reader (VICTOR3, Perkin Elmer, USA)
14. Multiwell plates (Corning, USA)

15. Polycarbonate membranes (100 nm) (Nuclepore, Whatman, USA)
16. Rotary evaporator (R 215, Buchi, Switzerland)
17. Shaking waterbath (Memmert, Germany)
18. Tissue culture flasks (Corning, USA)
19. Ultra-purifier water system (Maxima UF, England)
20. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)
21. Vortex mixer (G-560-E, Scientific industries, USA)
22. Water bath (OVE 14, Memmert, Germany)
23. Zetasizer (Zetasizer Nano series, Nano-zs, Malvern, UK)

Methods

1. Preparation of liposomes

1.1 Preparation of conventional liposomes

1.1.1 Preparation of calcein-loaded liposomes

Calcein-loaded liposomes were prepared by the dehydration-rehydration method (Walde et al., 2001). The total lipid concentration was 50 mg/mL. Alpha-tocopherol at 0.1 mol% was used as an antioxidant in all preparations. The liposomal preparation comprising soybean phosphatidylcholine (SPC) and cholesterol (CH) at the molar ratio of 70:30 was used for neutral liposomes. For the experiments to investigate the effect of liposomal surface charges, SPC, CH, and either stearylamine (SA) or dicetylphosphate (DCP) at the molar ratio of 60:30:10 were used for positively and negatively charged liposomes, respectively.

Briefly, blank liposomes were prepared first in Ultrapure® water by the film-hydration method. The lipid solution in chloroform (250 mg/10 mL chloroform) was introduced into a 100 mL round bottomed flask attached to a rotary evaporator. The solvent was evaporated at 40 °C under reduced pressure to complete dryness. The resultant lipid film was hydrated with 5 mL of Ultrapure® water at 40 °C with intermittent vortexing until liposome dispersion was formed. The liposome dispersion was subjected to freeze drying for approximately 30 hours, until a constant weight was obtained. Three batches of blank liposomes were prepared. The dried liposomes were kept refrigerated at -20 °C until use. Prior to each experiment, the obtained dried powder was rehydrated with calcein solutions (calcein 20 mM or 80 mM in 0.3 N NaOH) at 40 °C for at least 2.5 hours on a shaking water bath. A routine check of liposome dispersion for quality control was conducted under a light microscope at 400x magnification. The liposome dispersion was then extruded through 100 nm polycarbonate membranes with a hand-held extruder (LiposoFast™, AVESTIN, Canada) for 19 cycles in order to reduce liposome size to approximately 100 nm.

Non-associated calcein was separated from liposomes by gel filtration method using 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (pH 7.4) as the eluting fluid. The amount of calcein-encapsulated in liposomes was determined using a microplate reader at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. The calcein amount was back

calculated from the standard calibration line prepared for each assay. The amount of phospholipid in the preparation was determined by the standard Bartlett assay (New, 1989). The calcein-loaded liposomes were kept protected from light in a refrigerator and used within the same day of separation to avoid bleaching of the fluorescent marker and calcein leakage from liposome vesicles.

1.1.2 Preparation of calcein AM-loaded liposomes

Calcein AM-loaded liposomes were prepared by the film-hydration method (New, 1989) with the total lipid concentration of 35 mg/mL. The liposomal preparation composed of SPC and CH at the molar ratio of 70:30 was used for neutral liposomes. SPC, CH, and either SA or DCP at the molar ratio of 60:30:10 were used for positively and negatively charged liposomes, respectively. The alpha-tocopherol at 0.1 mol% was used as an antioxidant. Calcein AM (2.5 μM) was added with other lipid components in the lipid phase to form a lipid film. The preparation method was as follows. Briefly, all lipids were dissolved in chloroform at 17.5 mg total lipid/1 mL chloroform and the solution was transferred to a 100 mL round-bottomed flask. The organic solvent was evaporated under reduced pressure to form a thin lipid film using a rotary evaporator. All traces of the organic solvent were removed from the lipid film by keeping the flask under reduced pressure for another 1 hour. The lipid film was then hydrated with 10 mM HEPES buffer (pH 7.4) at 40 °C with intermittent vortexing for at least 2.5 hours. The resultant liposome dispersion was extruded through 100 nm polycarbonate membranes with a hand-held extruder for 19 cycles in order to reduce liposome size. The liposome dispersion was kept protected from light in a refrigerator until use. The preparation was used within two days.

1.2 Preparation of PEGylated liposomes (PEG liposomes)

1.2.1 Preparation of calcein-loaded PEG liposomes

SPC, CH, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2000-PE) at the molar ratio of 67:28:5 were used for neutral liposomes. SPC, CH, either SA or DCP, and PEG2000-PE at the molar ratio of 57:28:10:5 were used for charged liposomes. Alpha-tocopherol at 0.1

mol% was used as an antioxidant in all preparations. The total lipid concentration was 50 mg/mL.

Calcein-loaded PEG liposomes were prepared by the film-hydration method as described in Section 1.1.2 to economize the use of PEG2000-PE. The lipid film was then hydrated with calcein solutions (calcein 20 mM or 80 mM in 0.3 N NaOH) for at least 2.5 hours. The liposome dispersion was extruded and the non-associated calcein was removed by gel filtration as described in Section 1.1.1. The preparation was used within the same day.

1.2.2 Preparation of calcein AM-loaded PEG liposomes

Calcein AM-loaded PEG liposomes were also prepared by the film-hydration method as described in Section 1.1.2 with the total lipid concentration of 35 mg/mL. SPC, CH, and PEG2000-PE at the molar ratio of 67:28:5 were used for neutral liposomes. SPC, CH, either SA or DCP, and PEG2000-PE at the molar ratio of 57:28:10:5 were used for charged liposomes. The alpha-tocopherol at 0.1 mol% was used as an antioxidant. Calcein AM (2.5 μM) was added with other lipid components in the lipid phase to form a lipid film. The lipid film was hydrated with 10 mM HEPES buffer (pH 7.4) at 40 °C. The liposome dispersion was further extruded through 100 nm polycarbonate membranes to reduce the liposome size as described above. The preparation was used within two days.

Table 3 summarizes the compositions of all liposome preparations used in the study.

Table 3: The summary of liposome compositions used in the study

Liposome type	composition	ratio (mol%)
Conventional liposomes		
Neutral	PC:CH	70:30
Positive	PC:CH:SA	60:30:10
Negative	PC:CH:DCP	60:30:10
PEGylated liposomes		
PEG Neutral	PC:CH:PEG2000-PE	67:28:5
PEG Positive	PC:CH:SA:PEG2000-PE	57:28:10:5
PEG Negative	PC:CH:DCP:PEG2000-PE	57:28:10:5

2. Characterization of liposome preparations

2.1 Particle size and surface charge

The particle size and surface charge of liposomes were measured by dynamic laser light scattering using a Zetasizer system (Zetasizer Nano series, Nano-zs, Malvern, UK). The experiments were conducted at ambient temperature. Liposomes were diluted with HEPES buffer (pH 7.4) before measuring the particle size. The zeta potential of blank and calcein-loaded liposomes was measured using HEPES buffer (pH 7.4) as diluent. The serum-free DMEM was used as diluent for the zeta potential measurement of calcein AM-loaded liposomes.

2.2 Determination of calcein entrapment efficiency

Liposomes were prepared as described in Sections 1.1.1 and 1.2.1 and characterized in terms of % calcein entrapment. Briefly, calcein-loaded liposomes were diluted and dissolved with 1% Triton X-100 in 10 mM HEPES buffer (pH 7.4) for 45 min with intermittent vortexing. The container of the mixture was protected from light during the process by wrapping with aluminum foil. The solution was further diluted to the desired concentration range. Calcein was quantified using a microplate reader at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. Standard calibration line was constructed from calcein

solutions in 1% Triton X-100 in 10 mM HEPES buffer (pH 7.4) for each assay. The entrapment efficiency was determined from the following equation:

$$\text{Entrapment efficiency} = \frac{\text{The amounts of calcein entrapped in liposomes}}{\text{The amounts of calcein used in the formulation}} \text{ X } 100$$

The amount of calcein (μg) per mg lipid in all liposome types was also calculated to determine the amounts of liposomes and the corresponding calcein solution used in the uptake study.

The entrapment efficiency of calcein AM was not carried out since a practical assay method was not available. Calcein AM was used at a very low concentration in liposome preparations (around 5 mmol%). The marker was dissolved within the lipid phase during the liposome preparation. Thus, all calcein AM was expected to be associated with the liposomal bilayers due to its hydrophobic nature.

3. Cultivation of Caco-2 cells

Caco-2 cells were grown in standard Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% L-glutamine, 1% non-essential amino acid, 1% penicillin G-streptomycin and 10% fetal bovine serum at 37 °C in a humidified CO_2 incubator. At approximately 70-80% confluence, Caco-2 cells were subcultured routinely. Briefly, the cell monolayer was washed with phosphate buffered saline (PBS) and detached from the culture flask by incubating with 0.25% trypsin in 1mM EDTA solution for 3-5 min at 37 °C. Trypsin solution was replaced with the culture medium to stop the action of the enzyme and to disperse the cells by trituration. The dispersed cells were seeded at 5-6 x 10^5 cells per 15 mL in each 75-cm^2 culture flask.

For the experiments, cells were grown and trypsinized as described above. The cell suspension was seeded into 24-well culture plates at a concentration of 2.5 x 10^4 cells/0.5 mL/well. The cells were allowed to grow to form a monolayer and differentiate for 21 days. The medium was changed every two days until the start of the experiments.

4. Verification of P-gp function in Caco-2 monolayers

The uptake of calcein AM (25 nM), a P-gp substrate, in the presence and the absence of the P-gp inhibitor cyclosporin A (5 μM) into Caco-2 cells was compared to determine the function of P-gp. Briefly, Caco-2 cells in each well were washed with warm PBS and pre-incubated with serum-free DMEM at 37 °C for 30 min. After the pre-incubation period, the serum-free DMEM with or without cyclosporin A was replaced and incubated at 37 °C for another 30 min. After incubation with the P-gp inhibitor, the medium was then changed to calcein AM solution or calcein AM solution with cyclosporin A. The cells were further incubated at 37 °C for 30 min. At the end of incubation, the plates were placed on ice and cells in each well were washed with ice-cold PBS, followed by the addition of 1% Triton X-100 in 0.3 N NaOH to solubilize the cells. The cellular uptake of calcein AM was determined spectrofluorometrically by measuring the amount of calcein in the cells using a microplate reader with the excitation wavelength of 485 nm and the emission wavelength of 535 nm. The standard calibration line was prepared in 1% Triton X-100 in 0.3 N NaOH with untreated cells. The digested untreated cells were also used for background correction. The ratio of the amount of calcein in the presence and the absence of cyclosporin A was calculated as the accumulation enhancement factor (AEF) (Zastre et al., 2002).

5. Determination of MRP2 function in Caco-2 monolayers

The intracellular accumulation of calcein (8 μM), an MRP2 substrate, in the presence and the absence of the MRP2 inhibitor indomethacin (0.5 mM) in Caco-2 cells was determined to investigate the functional activity of MRP2 (Utoguchi et al., 2000). Briefly, Caco-2 cells in each well were washed with warm PBS and pre-incubated with serum-free DMEM at 37 °C for 30 min. The medium was then replaced with the serum-free DMEM with or without indomethacin and the cells were incubated at 37 °C for another 30 min. After incubation period, the medium was changed to calcein solution or calcein solution with indomethacin. The cells were further incubated at 37 °C for 30 min. At the end of incubation, the plates were placed on ice and cells in each well were washed with ice-cold PBS, followed by the addition of 1% Triton X-100 in 0.3 N NaOH to solubilize the cells. The cellular uptake of

calcein was determined by measuring the amount of calcein in the cells using a microplate reader with the excitation wavelength of 485 nm and the emission wavelength of 535 nm. The standard calibration line was prepared in 1% Triton X-100 in 0.3 N NaOH with untreated cells. The digested untreated cells were also used for background correction. The ratio of the amount of calcein in the presence and the absence of indomethacin was calculated as the AEF. The AEF was measured to corroborate the comparable activity of the efflux transporter MRP2 in all passages of Caco-2 cells used in this study.

6. Cell viability study (Freshney, 2005)

Caco-2 cells were treated with calcein/calcein AM-loaded liposomes and calcein/calcein-AM solution. The liposomes and the corresponding solutions were diluted with serum-free DMEM to the desired concentrations. After dilution, the lipid concentration was in the range of 0.175-1.4 mg/mL. The treatment times were 30, 60, 90, and 120 min. Serum-free DMEM was used as the control. After the treatment period, the treatment medium was removed and the cells were washed with warm PBS. The medium was then replaced with 0.5 mL of MTT solution (0.4 mg/mL in serum free DMEM). Cells were further incubated in the presence of MTT solution at 37 °C for 4 hours. The MTT reagent was removed at the end of incubation. The formazan crystals in the cells were dissolved in DMSO with constant shaking. The solution was diluted to an appropriate concentration and the amount of formazan production was quantified using a microplate reader at 570 nm. Cell viability was calculated as the percentage of the control.

7. Effects of liposomal composition on the uptake of model compounds into Caco-2 cells

7.1 Effect of liposomal composition on calcein uptake into Caco-2 cells

The effect of PEGylation and surface charge of liposomes on the uptake of calcein into Caco-2 cells was studied. The amounts of calcein uptake into Caco-2 cells were compared among liposomes with various compositions using the calcein solution at the corresponding calcein concentration as the control group.

Liposome preparations were prepared as described in sections 1.1.1 and 1.2.1. Caco-2 cells in culture plates were washed with pre-warmed PBS and pre-incubated with serum-free DMEM at 37 °C for 30 min. After the pre-incubation period, the serum-free DMEM was changed to calcein solution or calcein-loaded liposomes at a lipid concentration of 0.35 mg/mL in serum-free DMEM. The cells were incubated at 37 °C for another 30 min. At the end of incubation, the plates were placed on ice and cells in each well were washed with ice-cold PBS, followed by the addition of 1% Triton X-100 in 0.3 N NaOH to solubilize the cells. The uptake of calcein was determined spectrofluorometrically by measuring the amount of calcein in the cells using a microplate reader. The excitation wavelength was 485 nm and the emission wavelength was 535 nm. The standard calibration line was prepared in 1% Triton X-100 in 0.3 N NaOH along with untreated cells. Digested untreated cells were also used for background correction.

Protein content in each well was analyzed by the Bradford assay. The Bradford assay is based on the absorbance shift from 465 to 595 nm observed from the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The dye binds to protein resulting in a color change from a reddish brown to blue. The absorption is proportional to the protein amount. The protein determination was conducted in 96-well plates. Briefly, two fold dilutions of the protein samples were prepared. Five microliters of either protein standards, bovine serum albumin (BSA), or protein samples was added into separate wells, followed by the addition of the Bradford reagent (250 μL). The solution was then mixed and further incubated for 10 min. The absorbance was measured using a microplate reader at 595 nm within 60 min. The blank, 0.5% Triton X-100 in 0.15 N NaOH, was used for background correction. The protein concentration was determined by back-calculation from a calibration line of the standard BSA in the linear concentration range (0.1-1 mg/mL of protein) as recommended by the manufacturer of the assay kit.

7.2 Effect of liposomal composition on calcein AM uptake into Caco-2 cells

The effect of PEGylation and surface charge on the uptake of calcein AM into Caco-2 cells of liposomes was similarly studied. The amounts of calcein AM

uptake into Caco-2 cells were compared among liposomes with different compositions using calcein AM solution as the control group.

The liposome preparation method was as described in sections 1.1.2 and 1.2.2. The experiment was conducted using the same method as described in Section 7.1. Caco-2 cells were incubated with calcein AM solution or calcein AM-loaded liposomes at a lipid concentration of 0.35 mg/mL in serum-free DMEM at 37 °C for 90 min. Protein content in each well was also analyzed by the Bradford assay as described in Section 7.1.

8. Verification of the major mechanism of delivery of PEGylated liposomes into Caco-2 cells

Calcein was used as the fluorescent aqueous marker for liposomes at a self-quenching concentration (80 mM) and at a dequenching concentration (20 mM) (New, 1989). Cells were incubated with calcein solution or calcein-loaded liposomes at 37°C for 30 min. At the end of the incubation period, cells were washed with ice-cold PBS, detached from culture plates with 0.05% trypsin in 1 mM EDTA, and then suspended in 0.5 mL of PBS. The suspended cells were directly introduced into a flow cytometer (FACSCalibur, Becton Dickinson, USA) and analyzed under an argon ion laser with the excitation and emission wavelengths of 488 and 525 nm, respectively. At least 10,000 events were acquired and analyzed per sample. Untreated cells were used as the control. The fluorescence intensities of calcein-loaded liposomes and calcein solution were calculated as the percentage of the control and were further compared.

Furthermore, the uptake of calcein at both self-quenching and dequenching concentration was also measured using a microplate reader with the excitation wavelength of 485 nm and the emission wavelength of 535 nm after cell digestion as described in Section 7. The results from flow cytometric measurement and from cell digestion were compared to confirm the endocytosis mechanism.

9. Statistical analysis

All the experiments were repeated at least three times with three passages of the cells and three batches of liposomes unless stated otherwise. Statistical analysis

was performed using Student's t-test or One-way ANOVA with Tukey HSD or Dunnett T3 as a post hoc multiple comparison test. A p-value of less than 0.05 was considered statistically significant. The statistical analysis was performed on the SPSS Statistics Base 17.0 for Windows (SPSS serial no.: 5068054).

CHAPTER IV

RESULTS AND DISCUSSION

1. Physical properties of liposome preparations

1.1 Particle size and surface charge

1.1.1 Particle size

The liposome sizes estimated from dynamic light scattering are shown in Tables 4-6. The sizes of blank, calcein-loaded, and calcein AM-loaded liposomes were within a narrow range for each type of liposomes. In general, conventional liposome size slightly decreased in the presence of surface charge. Addition of PEG seemed to negate the effect of surface charge, resulting in a narrower size distribution between liposomes with and without charges. The statistical test results are displayed in Appendix A for clarity. A similar result has been previously reported by Zhao et al. (2007) with doxorubicin liposomes prepared by the emulsification-homogenization-diafiltration method.

Table 4: The particle size and surface charge of blank liposomes. Data are shown as mean ± SD (n = 3 batches).

Liposome type	Size (nm ± SD)	Zeta Potential (mV)
Neutral	117.2 ± 3.69	-16.9 ± 5.07
Positive	103.9 ± 0.81	43.8 ± 14.25
Negative	88.2 ± 5.32	-44.1 ± 7.58
PEG Neutral	109.5 ± 3.00	-11.3 ± 4.61
PEG Positive	112.4 ± 0.47	8.90 ± 1.01
PEG Negative	111.0 ± 0.36	-27.6 ± 6.73

Table 5: The particle size and surface charge of calcein-loaded liposomes. Data are shown as mean ± SD (n = 3 batches).

Liposome type	Size (nm ± SD)	Zeta Potential (mV)
Neutral	97.8 ± 2.78	-49.9 ± 3.68
Positive	92.4 ± 0.99	-34.0 ± 4.52
Negative	83.5 ± 1.87	-56.1 ± 6.01
PEG Neutral	97.9 ± 3.09	-30.1 ± 1.61
PEG Positive	100.0 ± 0.83	-30.0 ± 3.95
PEG Negative	96.6 ± 0.77	-33.5 ± 2.31

Table 6: The particle size and surface charge of calcein AM-loaded liposomes. Data are shown as mean ± SD (n = 3 batches).

Liposome type	Size (nm ± SD)	Zeta Potential (mV)
Neutral	126.4 ± 1.98	-3.22 ± 0.974
Positive	118.8 ± 1.40	5.87 ± 0.495
Negative	112.6 ± 2.29	-14.4 ± 0.850
PEG Neutral	126.1 ± 2.86	-1.71 ± 0.187
PEG Positive	120.6 ± 2.30	-0.80 ± 1.671
PEG Negative	117.0 ± 0.95	-1.52 ± 0.570

The significant difference in liposome size may result in the differences in the liposome-cell interaction (Liu, Mori and Huang, 1992; Uchiyama et al., 1995). Uchiyama and co-workers (1995) reported that liposomes with 59 nm in size were directly taken up by Yoshida sarcoma cells 2.5 times higher than liposomes larger than 100 nm. In another work, Liu et al. (1992) found that GM_1-containing liposomes with the size ranging from 70 to 200 nm showed the high uptake by EMT6 tumor in mouse, compared to those with the size outside this range. However, the difference in liposome-cell interaction in this study could not be attributed to the difference in size. As shown in Tables 5 and 6, the differences in liposome size among the conventional liposomes and the PEGylated liposomes were minimal.

1.1.2 Surface charge

The zeta potential values of blank liposomes are displayed in Table 4. Neutral liposomes showed a slightly negative surface charge. Generally, the negative value shown in neutral liposomes may come from impurities of phospholipids used in the preparations (Manconi et al., 2007). The zeta potential of charged liposomes displayed net negative or positive value due to the charge of either DCP or SA added to the preparation, respectively. Incorporating PEG in the formulation tended to reduce the zeta potential values in both charged and uncharged liposomes. PEG incorporated in the liposome formulations is known to shield either negative or positive charge on the surface of liposomes, resulting in less-negative or less-positive zeta potential (Vanić et al., 2012).

On the contrary, net negative zeta potential values were found in all types of calcein-loaded liposomes (Table 5). This may be resulted from the adsorption of the negatively charged calcein on the surface of liposomes. For conventional liposomes, the rank of the surface charge density on liposomes was in the following order: negatively charged > neutral > positively charged. Addition of PEG on liposome surface resulted in less negative zeta potential values in all liposome types due to the shielding effect. It is worth noting that the zeta potential values in all types of PEGylated calcein-loaded liposomes were rather similar.

The zeta potential values of calcein AM-loaded liposomes are shown in Table 6. For conventional liposomes, a small negative zeta potential value was seen in neutral liposomes. Charged liposomes showed net slightly negative or positive zeta potential values due to the charge of either DCP or SA in the formulation, respectively. Incorporation of PEG in the calcein AM-loaded liposomes into both charged and uncharged ones resulted in all slightly negative zeta potential values due to the shielding effect. As with calcein-loaded liposomes, the difference in the surface charge in all PEGylated calcein AM-loaded liposomes was also minimal. However, the numerical zeta potential values of calcein AM-loaded liposomes should not be compared quantitatively with those of blank and calcein-loaded liposomes due to the disparity in the experimental conditions (See Section 2.1 in Materials and Methods). Measurement of zeta potential of calcein AM-loaded liposomes could not be carried out with HEPES buffer. The preliminary experiments with HEPES buffer on different

zeta potential measuring machines consistently resulted in charring of the samples. The reason behind this finding was unknown, even to the experts of the manufacturer of the instrument. However, the charring was not seen with other liposome samples or when calcein AM-loaded liposomes were diluted with the serum-free medium. The concentrations of liposomes used were practically the same in all measurements. This problem was not found when the instrument was set to the size measuring mode.

1.2 Entrapment efficiency of calcein-loaded liposomes

Determination of calcein entrapment efficiency was crucial for the uptake study of calcein-loaded liposomes into Caco-2 cells. Since calcein is a hydrophilic compound, only a certain amount of calcein could be entrapped in liposome vesicles. Most of calcein would reside as free calcein in the vehicle. To avoid any confounding results from the free calcein, it needed to be removed. The quantification of the exact amount of calcein entrapped in liposomes was essential for the uptake comparison between calcein-loaded liposomes and calcein solution, where comparable calcein amount was required for each preparation. In contrast, the entrapment efficiency of calcein AM, a lipophilic compound, was not necessary. Calcein AM was used in such small amounts compared to the amount of total lipid (approximately 5 mmol%). All calcein AM used in the preparation was expected to reside within the bilayer of liposomes. Thus, the amount of calcein AM entrapped was considered equal in all liposome preparations. The entrapment efficiency was experimentally determined only for liposomes containing calcein.

The calcein entrapment of each liposome preparation is shown in Table 7. For hydrophilic compounds, it is known that the entrapment correlates with the vesicle size. The entrapment of a hydrophilic compound can be used to estimate the liposome size, provided that the composition is comparable (Torchilin and Weissig, 2003). However, the differences in the entrapment efficiency seen with various types of liposomes in this study could not be attributed to such a small difference in liposome size among these liposomes (Table 5).

Table 7: Calcein entrapment efficiency of various liposome formulations. Data are shown as mean ± SD (n = 12-15 batches).

Liposome type	Calcein entrapment efficiency (%) (mean ± SD)
Neutral	4.45 ± 0.64
Positive	6.90 ± 0.26
Negative	2.75 ± 0.23
PEG Neutral	1.24 ± 0.16
PEG Positive	3.97 ± 0.28
PEG Negative	0.68 ± 0.04

The higher calcein entrapment was found with liposomes containing positive charges for both conventional and PEGylated liposomes ($p < 0.05$). These findings might be caused by the electrostatic attraction between the carboxylate anion of calcein and the protonated amine group of SA on the membrane of liposomes (see Appendix B and C). Calcein molecules would both dissolve in the aqueous core of the liposome vesicle and associate with the liposome membrane of the positively charged liposomes, resulting in the higher entrapment. As expected, incorporation of DCP reduced calcein entrapment, presumably from the electrostatic repulsion between the negatively charged calcein and the anionic headgroup of DCP. The similar effect of liposome surface charge on entrapment of ionizable hydrophilic molecules has been reported for many compounds (Bai, Gupta and Ahsan, 2009; Gharib, Faezizadeh and Godarzee, 2012; Mohammed et al., 2004). The electrostatic interaction can be an effective means to increase drug entrapment in liposomes.

The presence of PEG in the formulations decreased the amount of calcein entrapped in the liposomes in comparison with the corresponding conventional liposomes. This may be explained partly by the space effect. Since the PEG chains would also be located inside the aqueous compartment of liposomes, a smaller space would be left available for calcein in the aqueous compartment. This result was in good agreement with a previous report where calcein entrapment efficiencies were

compared in phosphatidylethanolamine-based liposomes with and without PEGylation (Vanić et al., 2012).

The amount of calcein per mg lipid in all liposome types was also calculated. The means and standard deviations from 3 representative batches of liposomes and the ranges from all liposome batches are shown in Table 8. The calcein entrapment values normalized for total lipid were used to calculate the amount of liposomes and the corresponding calcein for cell viability study, uptake study and in the elucidation of mechanism of liposome uptake into Caco-2 cells. Since the total lipid was kept constant in these experiments, the amount of calcein solution was adjusted according to the entrapment efficiency of each liposome type. Table 8 shows that the entrapment efficiency for each type of liposomes was within a narrow range, indicating the reproducibility of the preparation process.

Table 8: The amount of calcein per mg lipid of various liposome formulations. Data are shown as mean ± SD (n = 12-15 batches).

Liposome type	**Calcein (µg)/mg lipid (mean ± SD)**
Neutral	11.23 ± 1.46
Positive	17.87 ± 1.40
Negative	6.64 ± 0.69
PEG Neutral	3.00 ± 0.56
PEG Positive	11.80 ± 1.90
PEG Negative	1.88 ± 0.23

2. P-gp and MRP2 functions in Caco-2 monolayers

2.1 P-gp function in Caco-2 monolayers

The aim of this study was to confirm that Caco-2 monolayers used in the experiment could express functional P-gp. The uptake of the P-gp substrate calcein AM has been used to evaluate the functional P-gp (Bauer, Miller and Fricker, 2003; Legrand et al., 1998; Varma, Sateesh and Panchagnula, 2004). Due to its lipophilicity,

calcein AM can permeate across the cell bilayer by passive diffusion. The ester bonds are then cleaved by cellular esterases and the highly fluorescent calcein is produced. Calcein, a hydrophilic compound, is trapped inside the cells. When P-gp is inhibited, calcein AM efflux decreases, resulting in higher intracellular accumulation of calcein. The calcein amount detected by spectrofluorometry reflects the intracellular accumulation of calcein AM. Cyclosporin A has been widely used as a P-gp inhibitor (Legrand et al., 1998; Luker et al., 1997; Morjani and Madoulet, 2010; Qadir et al., 2005; Sun et al., 2008). It has also demonstrated activity as a modulator of multidrug resistance-associated protein-1, breast cancer resistance protein, and lung resistance protein (Qadir et al., 2005). The modulatory effect of cyclosporin A on calcein AM uptake has been used to assess the function of P-gp in various types of cells (Legrand et al., 1998; Utoguchi et al., 2000).

The activity of P-gp in Caco-2 cells is known to be cell passage dependent (Sambuy et al., 2005; Yu, Cook and Sinko, 1997). In this study, the intracellular accumulation of calcein AM in the present of cyclosporin A was increased with the average accumulation enhancement factor (AEF) of 2.77 (Appendix D). The AEF of Caco-2 cells in the cell passage number between 37 and 66 were not significantly different, ranging from 2.64 to 2.99. These values were in good agreement with the range reported in the literature (from 1.5 to 2.5), where the functional activity of P-gp was determined by the uptake study (Eneroth et al., 2001; Legrand et al., 1998; Utoguchi et al., 2000; Zastre et al., 2002). These results confirmed that Caco-2 cells used in the experiments expressed functional P-gp. The only slightly differences in the AEF ensured that these monolayers were suitable as a model for comparing the interaction among various liposome formulations with the cells. The AEFs among different laboratories were reported to be varied. The functional activity of P-gp may be affected by cell origin, culture condition, passage number, and also trypsinization (Elsby et al., 2008; Sambuy et al., 2005).

2.2 MRP2 function in Caco-2 monolayers

MRP2 is another efflux transporter located on the apical side of Caco-2 monolayers (Lin et al., 2011; Sun et al., 2008). It is one of the efflux transporters that influence drug absorption and also cause MDR in tumor cells. Many substances have

shown to be substrates of MRP2 such as vinblastin, methotrexate, irinotecan, talinolol, oestradiol 17β-glucuronide, quercetin, and calcein (Elsby et al., 2008; Li et al., 2008; Sun et al., 2008; Utoguchi et al., 2000). To study the uptake and the transport of MRP2 substrates, several compounds including indomethacin, probenecid, valproic acid, and glibenclamide can be used as MRP2 inhibitors (Li et al., 2008; Utoguchi et al., 2000).

The aim of this study was to ascertain that Caco-2 monolayers used in the experiment did not express significant differences in functional activity of MRP2 as the cell passage changed. Since calcein used in the study is a substrate of MRP2 (Essodaïgui, Broxterman and Garnier-Suillerot, 1998), interpretation of the results with calcein or calcein AM could be confounded by MRP2 activity. The intracellular accumulation of calcein with or without indomethacin was compared to detect MRP2 activity in Caco-2 cells. The AEFs in Caco-2 cells used (passage numbers of 37-66) were within the range of 1.47-1.69 (Appendix D). The result indicated that Caco-2 cells used in all experiments did not show any significant differences in MRP2 functional activity. Thus, the differences in liposome-cell interaction in this study would be influenced by liposome compositions rather than by P-gp and MRP2 functions.

4. Cell viability study

The absence of toxicity is an essential property for any drug delivery systems. The cell viability assay was also crucial in order to confirm that the differences in the uptake efficiency of both calcein and calcein AM into Caco-2 cells were not associated with the toxicity of liposomes. Cell viability greater than 90% was considered to be non-toxic to the cells in this study.

4.1 Cell viability of Caco-2 cells treated with calcein-loaded liposomes

The cell viability from the treatments of Caco-2 cells with calcein solution and calcein-loaded liposomes is illustrated in Figure 4. Cells were treated with calcein solution at 30 μg/mL of calcein as a reference. The calcein concentration corresponded with the amount of calcein from the conventional positively charged liposomes at 1.4 mg/mL total lipid. This type of liposomes showed the highest entrapment efficiency and thus would expose the cells to the highest calcein

concentration. The calcein-loaded liposomes were tested at various lipid concentrations (0.175-1.4 mg/mL of total lipid) for 60 min. The incubation period was selected from a preliminary study (Appendix E). Cell viability was calculated as the percentage of the untreated control cells. The results showed that calcein solution and all liposome treatments with the lipid concentrations from 0.175-0.7 mg/mL were not toxic to the cells. Cell viability was 93% with calcein solution. Cells incubated with conventional liposomes at all tested lipid concentrations showed cell viability between 91% and 106%. Cell viability was in the range of 94-103% in cells treated with PEGylated liposomes at total lipid concentrations of 0.175-0.7 mg/mL. On the other hand, cells treated with PEGylated liposomes at 1.4 mg/mL of total lipid displayed a dramatic decrease in cell viability (34%, 8%, and 32% from PEG neutral, PEG positively charged, and PEG negatively charged liposomes, respectively). Consequently, liposomes were used in this study at concentrations below this concentration. The cytotoxicity of PEGylated liposomes at higher lipid concentration has not been reported elsewhere. However, most studies from PEGylated liposomes in the literature used the PEGylated liposomes at relatively much lower concentrations (Miller et al., 1998; Sugiyama and Sadzuka, 2013; Vanić et al., 2012; Zhao et al., 2007). The reason for the high toxicity of PEGylated liposomes to Caco-2 cells was not investigated further in this study.

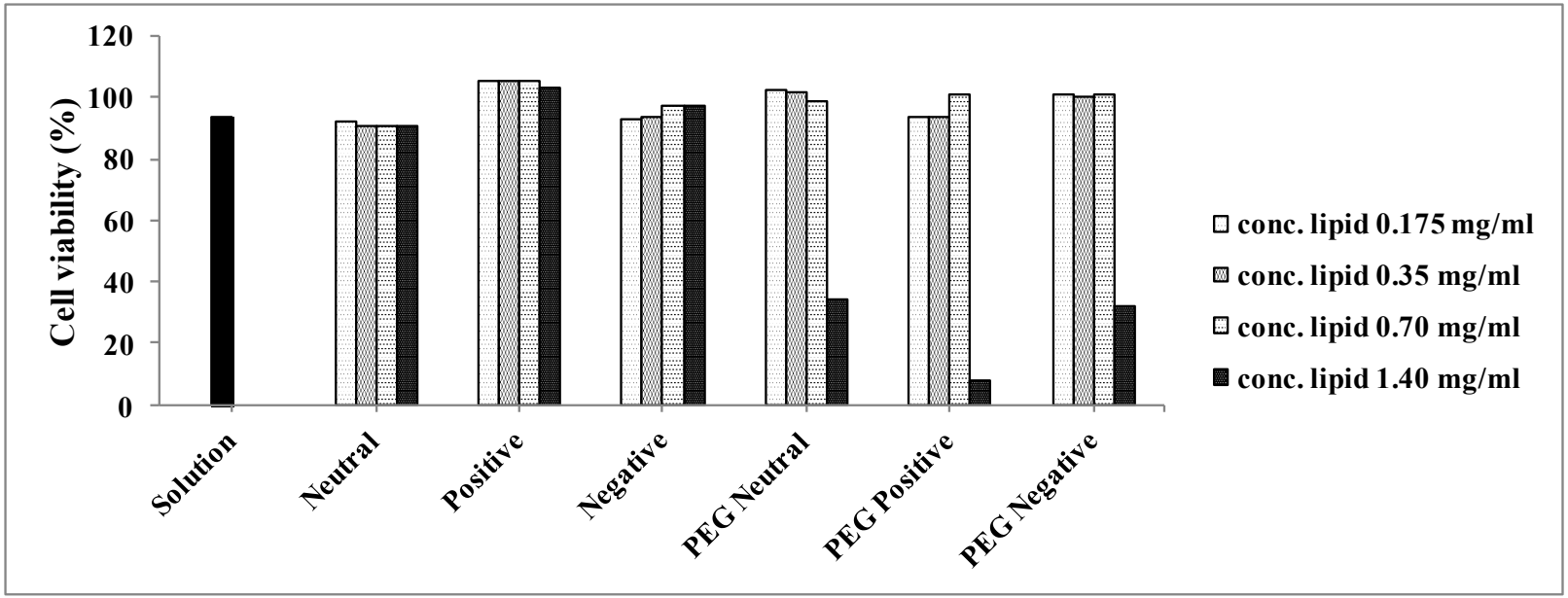

Figure 4: Cell viability measured by MTT assay of Caco-2 cells treated with calcein solution (30 µg/mL) and calcein-loaded liposomes at various lipid concentrations for 60 min. Data are shown as the average values of data from 3 wells. The study was done with one batch of each liposome type in 2 passages of Caco-2 cells.

4.2 Cell viability of Caco-2 cells treated with calcein AM-loaded liposomes

The results from cell viability study with calcein AM solution and calcein AM-loaded liposomes are shown in Figure 5. In this study, cells were incubated with calcein AM solution (25 nM) and calcein AM-loaded PEGylated liposomes at the lipid concentration of 0.35 mg/mL for 90 min. The lipid concentration of 0.35 mg/mL was selected deliberately in accordance with the concentration used in a previous study from this research group (Ing-orn Prasanchaimontri, 2009). The result in Section 4.1 also confirmed that PEGylated liposomes at this concentration would not be toxic to the cells. The incubation period was also selected from a preliminary study (Appendix E). The cell viability was more than 90% in all treatments (105% for calcein AM solution and 100%, 90%, and 110% for PEG neutral, PEG positively charged, and PEG negatively charged liposomes, respectively). Cytotoxicity of calcein AM-loaded conventional liposomes to Caco-2 cells has been previously studied in this research group (Ing-orn Prasanchaimontri, 2009). It was reported that calcein AM-loaded liposomes with similar compositions of those used in this present study were not toxic to the cells at the lipid concentration used. Thus, the experiments with the calcein AM-loaded conventional liposomes were not repeated in this study.

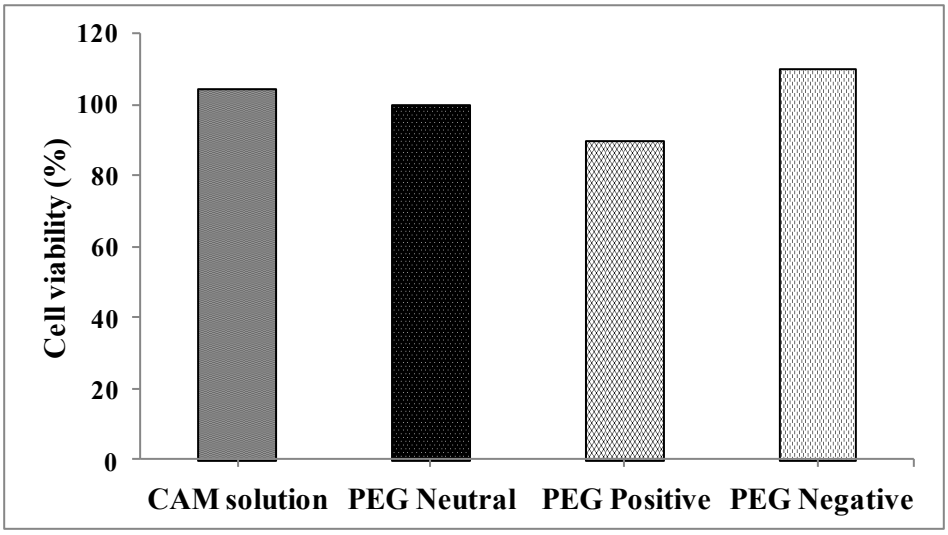

Figure 5: Cell viability measured by MTT assay of Caco-2 cells treated with calcein AM solution (25 nM) and calcein AM-loaded PEG liposomes at the lipid concentration of 0.35 mg/mL for 90 min. Data are shown as the average values from 3 wells in 1 passage of cells with 1 batch of liposomes.

5. Effects of liposomal composition on the uptake of model compounds into Caco-2 cells

5.1 Effect of liposomal composition on calcein uptake into Caco-2 cells

The preliminary study indicated that the uptake of calcein into Caco-2 cells from calcein-loaded neutral conventional liposomes was higher than that from calcein solution at all incubation periods (30, 60, 90, and 120 min) (Appendix E). The difference in the extents of calcein uptake from liposomes and from solution was unambiguous even at the incubation time of 30 min. Thus, the incubation period of the liposome formulations and the corresponding calcein solutions with Caco-2 cells was fixed at 30 min in all uptake studies.

The calcein uptake from various liposome formulations and the corresponding solutions is displayed in Figure 6. The results indicated that the interaction of liposomes with Caco-2 cells was affected by both liposome surface charge and PEGylation. Positively charged liposomes were the most efficient in the delivery of calcein into Caco-2 cells for both conventional and PEGylated liposomes. This observation could be attributed to the electrostatic interaction between the

positive charge on the liposome membrane and the negative charge of Caco-2 cell surface (Iseki et al., 1997). It is worth noting that the high cell-associated calcein fluorescent intensity observed with the liposomes carrying the positive charge may also be due to calcein in the vesicles adhered to the cell surface, not just the internalized dye molecules. The neutral and the negatively charged conventional liposomes displayed less cell-associated calcein fluorescent intensity than the positively charged conventional liposomes. Nevertheless, they were still successful in delivering calcein into the cells compared to the solutions.

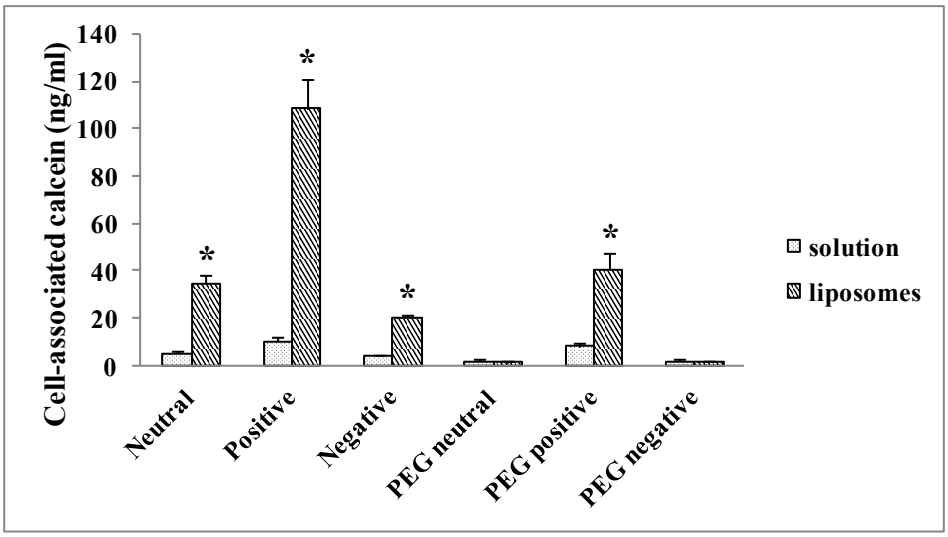

Figure 6: Cell-associated calcein in Caco-2 cells incubated with calcein-loaded liposomes with the lipid concentration of 0.35 mg/mL and calcein solutions at the corresponding calcein concentration for 30 min. Data are shown as mean ± SEM (n = 3 batches). * $p < 0.05$, compared with the corresponding solution

In addition, the calcein cellular uptake from the liposomes could be dependent on the entrapment efficiency (Table 7). For conventional liposomes, the efficiency of calcein delivery to Caco-2 cells decreased in the rank order of positively charged, neutral, and negatively charged liposomes, respectively. These calcein uptake results correlated well with the entrapment efficiency. For PEGylated liposomes, only PEG positively charged liposomes showed greater cell-associated

calcein than the corresponding solution which may be resulted from the high entrapment efficiency (see Table 7) and the electrostatic interaction. There was a lack of superior uptake of calcein from the neutral and negatively charged PEGylated liposomes. This could be explained partly by the low entrapment efficiency of the two formulations as well as the lack of interaction between these liposomes and the cells due to the steric shielding of the PEG chains.

Figure 7 was re-plotted from the data in Figure 6 for clarity. It shows that the accumulation of calcein from all conventional liposomes was higher than that from the PEGylated liposomes. The close contact between the liposomes and the cells is thought to be hindered by the hydration of the PEG moieties on the membrane of the PEGylated liposomes (Chan et al., 2012; Miller et al., 1998; Vanić et al., 2012). Without the close contact between and cells, adhesion and internalization of the vesicles cannot take place.

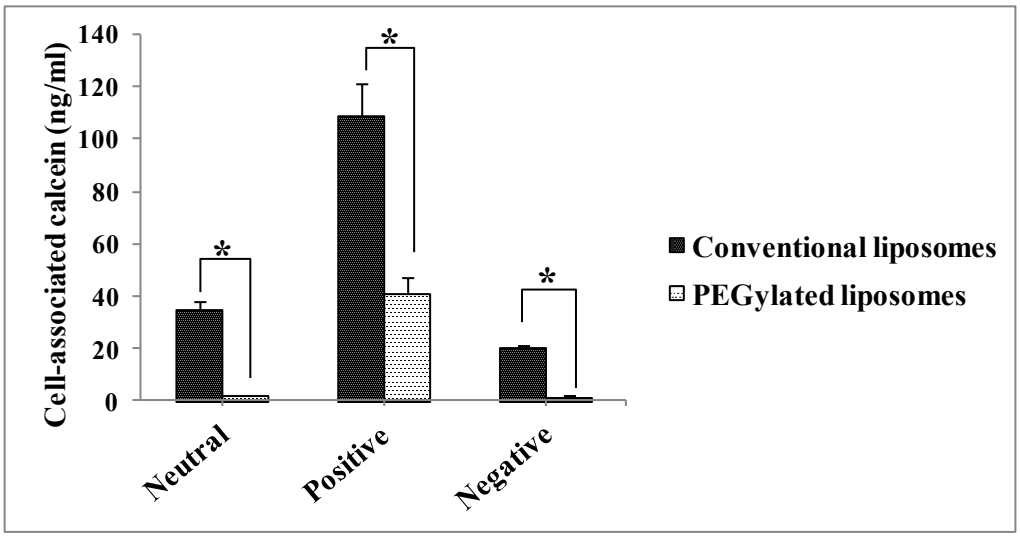

Figure 7: Cell-associated calcein in Caco-2 cells incubated with calcein-loaded conventional and PEGylated liposomes with the lipid concentration of 0.35 mg/mL for 30 min. Data are shown as mean ± SEM (n = 3 batches). * $p < 0.05$, compared with conventional liposomes

5.2 Effect of liposomal composition on calcein AM uptake into Caco-2 cells

The preliminary study also indicated that the cellular uptake of calcein AM from liposomes was higher than that from solution at all incubation times (30, 60, 90, and 120 min). However, the amounts of calcein detected after incubation with calcein AM solution and calcein AM encapsulated in both conventional and PEGylated neutral liposomes were clearly seen at 90 and 120 min (Appendix E). Thus, the uptake study of calcein AM from various liposome formulations was conducted with the incubation period of 90 min.

The calcein AM uptake from various liposome formulations and calcein AM solution is shown in Figure 8. The results also indicated that the interaction of liposomes with Caco-2 cells was affected by both liposome surface charge and PEGylation. Calcein AM from conventional neutral, PEGylated neutral, and PEGylated positively charged liposomes was taken up by Caco-2 cells more efficiently than that from the calcein AM solution. Though the negative charge on liposome surface has been reported to trigger endocytosis in HeLa cells (Manconi et al., 2007), such effect was not seen here. The neutral liposomes seemed to be taken up by Caco-2 cells much better than the negatively charged liposomes in both conventional and PEGylated liposomes. The discrepancy seen might be attributed partly to the difference in cell type (Bajoria, Sooranna and Contractor, 1997; Lee, Hong and Papahadjopoulos, 1992; Manconi et al., 2007; Miller et al., 1998). Positively charged conventional liposomes failed to improve the delivery of calcein AM into Caco-2 cells in comparison with calcein AM solution, in agreement with previous data (Ing-orn Prasanchaimontri, 2009). This may probably be due to a strong binding between the positive charge on the liposome membrane and the negative charge of Caco-2 cell surface. Positively charged vesicles may rapidly adhere onto the cell surface without internalization (Manconi et al., 2007). Less calcein AM liposomes would be available at the endocytic sites. The non-fluorescent calcein AM could not convert to the fluorescent calcein without internalization since the conversion requires esterases in the cells. This reasoning also corroborated well with the opposite results seen with the calcein liposomes above. With calcein liposomes, less non-specific binding was expected since the liposome surface was negatively

charged in all formulations regardless of the charged lipid present (see Table 5). In addition, the fluorescence would be detected even when the dye was still entrapped within the vesicles adhered to the cell surface. With the PEGylated positively charged liposomes, however, less non-specific cell adherence might take place due to the steric hindrance, resulting in a higher uptake.

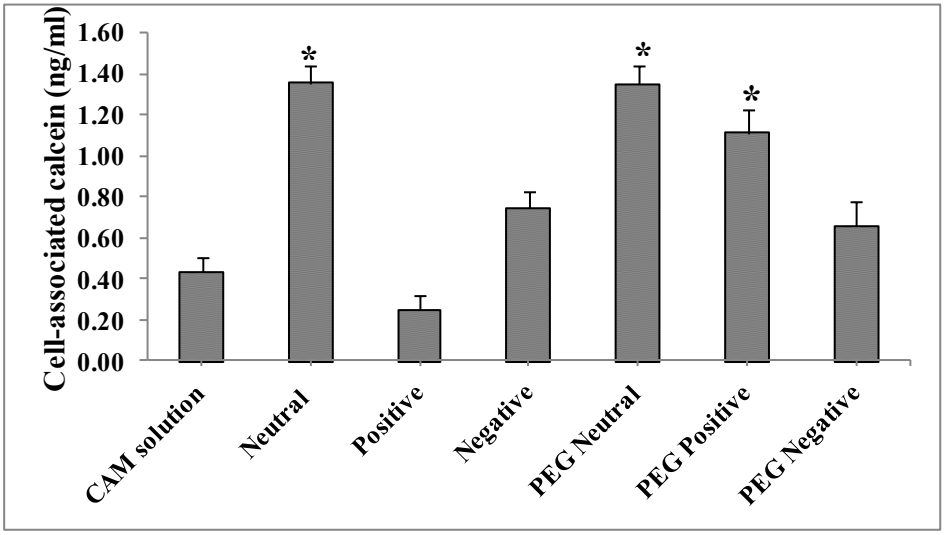

Figure 8: Cell-associated calcein in Caco-2 cells incubated with calcein AM-loaded liposomes with the lipid concentration of 0.35 mg/mL and calcein AM solution (25 nM) for 90 min. Data are shown as mean ± SEM (n = 3 batches). * $p < 0.05$, compared with the solution

For clarity, the effect of PEGylation on calcein AM uptake from liposomes is illustrated in Figure 9. The extents of cell-associated calcein were comparable for both neutral and negatively charged liposomes, implying similar liposome uptake into the cells. This estimation would be valid only when the mechanism of calcein AM delivery was via endocytosis, which was further investigated. The effect of PEGylation on cellular uptake of the positively charged liposomes has already been discussed above.

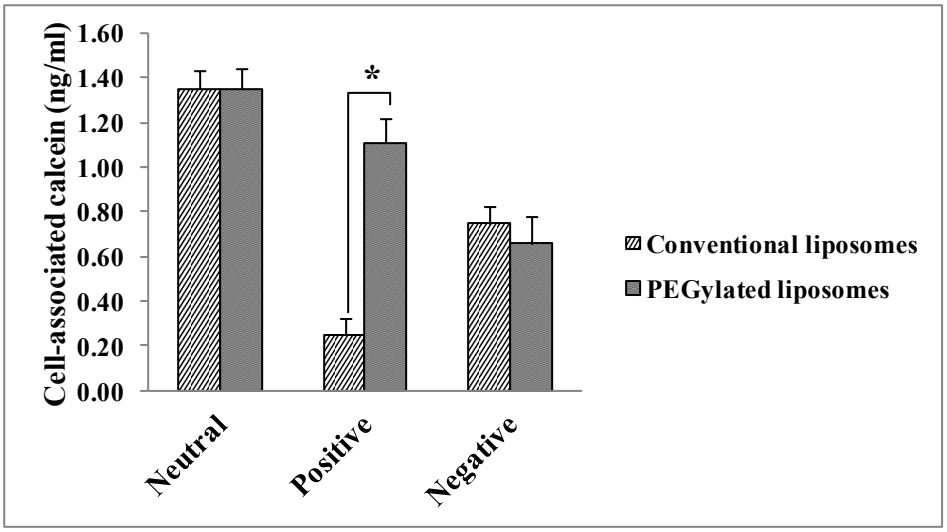

Figure 9: Cell-associated calcein in Caco-2 cells incubated with calcein AM-loaded conventional and PEGylated liposomes with the lipid concentration of 0.35 mg/mL for 90 min. Data are shown as mean ± SEM (n = 3 batches). * $p < 0.05$, compared with the corresponding PEGylated liposomes

In the study of calcein and calcein AM uptake into Caco-2 cells, protein content in each well was analyzed by the Bradford assay. It was found that the differences in protein contents in each well were within an acceptable limit (%CV < 15). Thus, the results from calcein and calcein AM uptake by Caco-2 cells seen here were resulted only from liposome compositions, not from the differences in the amount of cells in each well. The differences in the uptake of calcein and calcein AM encapsulated in various types of liposome seemed to be primarily due to the differences in the binding between liposomes and cells. The uptake of liposomes is known to depend on both surface properties of liposomes and types of cell (Bajoria et al., 1997; Chan et al., 2012; Lee et al., 1992; Miller et al., 1998). In one study, the effect of liposome surface charge on conventional and PEGylated liposomes was performed in two different cell lines: a human ovarian carcinoma cell line (HeLa) and a murine derived mononuclear macrophage cell line (J774) (Miller et al., 1998). The uptake of liposomes with similar compositions by these two cell types was different. In HeLa cells, the positively charged liposomes resulted in greater uptake than the neutral and the negatively charged liposomes for both conventional and PEGylated

liposomes. The uptake from PEGylated liposomes was also obviously lower than that from conventional liposomes. On the other hand, the greater interaction of liposomes with J744 cells was observed with both types of the charged liposomes than with the uncharged liposomes. Surface property of liposomes due to different types of phospholipid used also affects the uptake profile. In one study, the addition of negatively charged phospholipids including phosphatidylserine, phosphatidylglycerol, and phosphatidic acid on liposomes composed of egg PC and CH promoted the uptake by CV1 cells (an African green monkey kidney cell line). However, the addition of different anionic phospholipids namely monosialoganglioside GM_1, phosphatidylinositol, and PEG-PE did not promote the uptake by this cell line (Lee et al., 1992). Another study revealed that the anionic and neutral liposomes were successful in the delivery of carboxyfluorescein into trophoblast cells, probably by endocytosis (Bajoria et al., 1997).

Effect of PEGylation on the liposome uptake has also been investigated in several studies (Chan et al., 2012; Jung et al., 2009; Miller et al., 1998; Wang et al., 2010). Chan et al. (2012) reported that the transfection efficiency of PEGylated cationic liposomes-DNA complexes in mouse fibroblast L-cells was less than those without PEG. The transfection efficiency also associated with the amount of PEG-lipid, that is, the complexes with PEG at 5 mol% showed superior transfection efficiency than those with PEG at 10 mol% in both acid-labile and acid-stable PEG-lipids. Another study reported that PEG-coated polymeric liposomes resulted in higher calcein taken up by MCF-7 cancer cells than those without PEG (Wang et al., 2010). Furthermore, the structural lipid may also have some influence on the liposome-cell interaction. PEG-complexed cationic liposomes were reported to demonstrate superior intracellular uptake over Doxil®, the PEGylated phospholipid-based liposomes, in B16F10 cells (Jung et al., 2009). In this present study, the effect of PEGylation was also clearly seen in the uptake of both the hydrophilic calcein and the hydrophobic P-gp substrate calcein AM in Caco-2 cells.

6. Mechanism of PEGylated liposome uptake by Caco-2 cells

Liposomes and other particulate carriers can deliver their contents to the cells by various mechanisms (New, 1989; Torchilin and Weissig, 2003). Endocytosis is

usually the major mechanism proposed for liposomes (Chebbi et al., 2010; Miller et al., 1998; Un et al., 2012; Ziello, Huang and Jovin, 2010). To verify the major mechanism of the delivery of PEGylated liposomes that were promising for the delivery of low permeability compounds, calcein was used as the fluorescent aqueous marker for liposomes at a self-quenching concentration (80 mM) and at a dequenching concentration (20 mM). The fluorescence intensities associated with Caco-2 cells from calcein-loaded liposomes and calcein solution at both self-quenching and dequenching concentrations were detected by flow cytometry. To allow verification of the mechanism, fluorescence intensities were also determined by the spectrofluorometric method after cell digestion.

Conventional liposomes

From the uptake study, neutral liposomes showed the successful delivery of both calcein and calcein AM into Caco-2 cells. Thus, they were selected as the positive control to confirm the uptake mechanism. Conventional liposomes are known to be taken up by Caco-2 cells via endocytosis from several previous reports (Anderson et al., 2001; Bajoria et al., 1997; Chebbi et al., 2010; Miller et al., 1998;), including those from this research group (Araya Lukanawonakul, 2005; Ing-orn Prasanchaimontri, 2009). Fluorescence histograms from the flow cytometric study are shown in Figure 10 and the mean fluorescence intensity calculated as % of the control is summarized in Table 9.

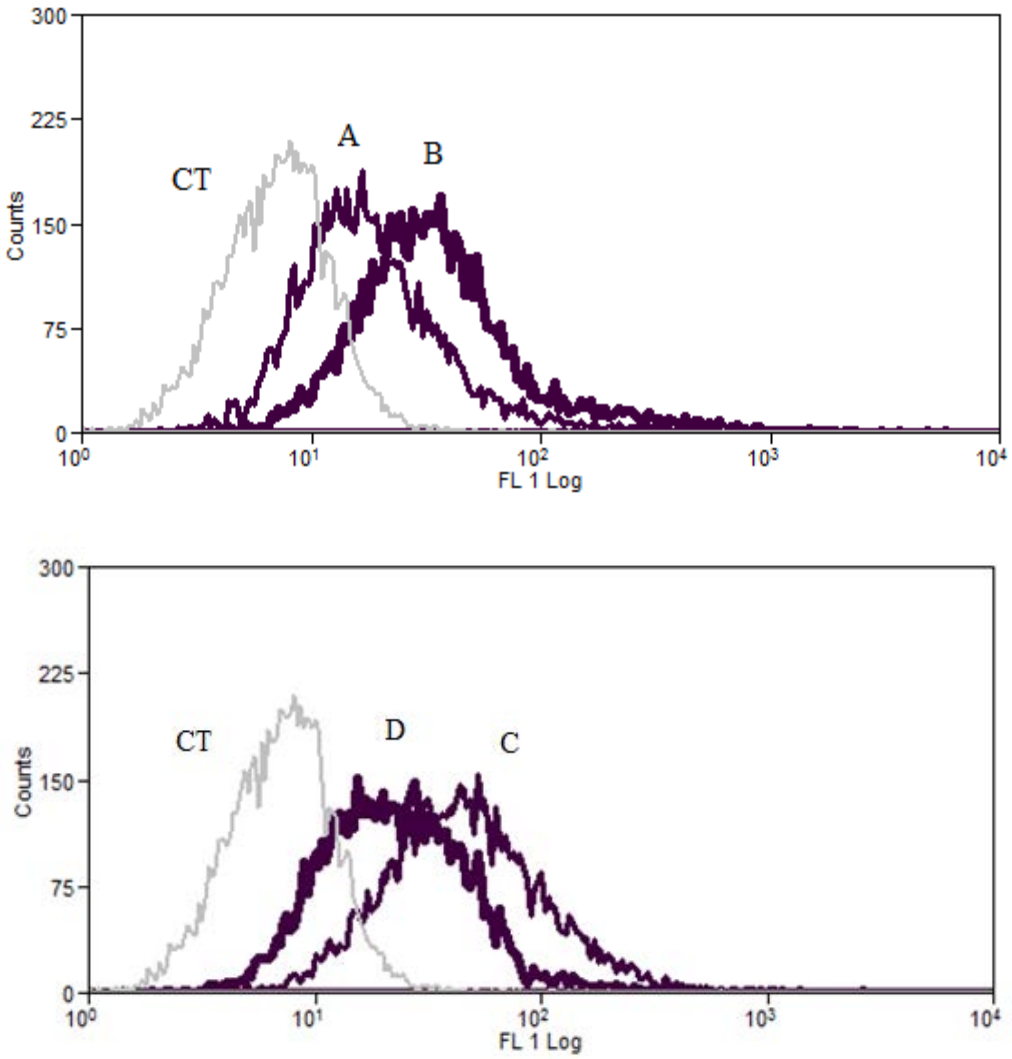

Figure 10: Fluorescence histograms from Caco-2 cells incubated with A) calcein solution at a dequenching concentration, B) neutral liposomes entrapping calcein at a dequenching concentration, C) calcein solution at a self-quenching concentration, and D) neutral liposomes entrapping calcein at a self-quenching concentration. Cells treated with serum-free DMEM were use as control (CT).

Table 9: Fluorescence intensity (% of control) of cell-associated calcein from the calcein solution and from the neutral liposomes measured by the flow cytometric method. Data are shown as % of control ± SEM (n = 3 batches).

	Fluorescence intensity (% of control)	
	Treatment with calcein at a dequenching concentration	Treatment with calcein at a self-quenching concentration
Solution	303.50 ± 9.47	922.30 ± 30.79
Neutral liposomes	808.91 ± 32.42	414.60 ± 8.65

The flow cytometric results showed that the fluorescence intensity of calcein from the liposomes was significantly greater than that from the solution at a dequenching concentration of calcein (20 mM) ($p < 0.05$). On the contrary, the fluorescence intensity of calcein at a self-quenching concentration (80 mM) from the liposomes was significantly less than that from the solution ($p < 0.05$). These results could be used to verify that the mechanism of delivery of conventional liposomes into Caco-2 cells is endocytosis, not fusion. At the dequenching concentration of calcein, liposomes showed higher fluorescence intensity compared to the solution due to the better cellular uptake of the hydrophilic dye entrapped in liposomes via endocytosis. The result from the experiment at the self-quenching concentration was also consistent with endocytosis, where liposomes containing calcein at the self-quenching concentration were confined within endosomes/lysosomes. Calcein at a self-quenching concentration gave only a little fluorescent intensity, less than that from the solution. It would have displayed strong fluorescence if liposomes had been taken up into the cells by fusion since the dye would have been diluted several fold and became less quenched after it was delivered to the cell cytoplasm. This fluorescence dequenching technique is well accepted as a method to distinguish between different liposome-cell interactions (New, 1989).

To corroborate the above findings further, the Caco-2 cells were treated under the same condition and the cells were digested with 1% Triton X-100. The extent of calcein was determined using a microplate reader. The results illustrated that cell-associated calcein from liposomes at both dequenching (20 mM) and self-

quenching (80 mM) concentrations was significantly higher than that from solution (Figure 11). Under this condition, calcein at the self-quenching concentration was released from the endosomes/lysosomes upon digestion with the surfactant and diluted by many hundred-fold in the reaction mixture. Thus, a dramatic increase in the fluorescence intensity from liposome treatment at both calcein concentrations was detected. As expected, liposomes at the self-quenching calcein concentration yielded much higher fluorescence intensity than liposomes at the dequenching calcein concentration. The disparity in fluorescence intensity agreed well with the difference in the total amount of calcein entrapped in these two liposome formulations.

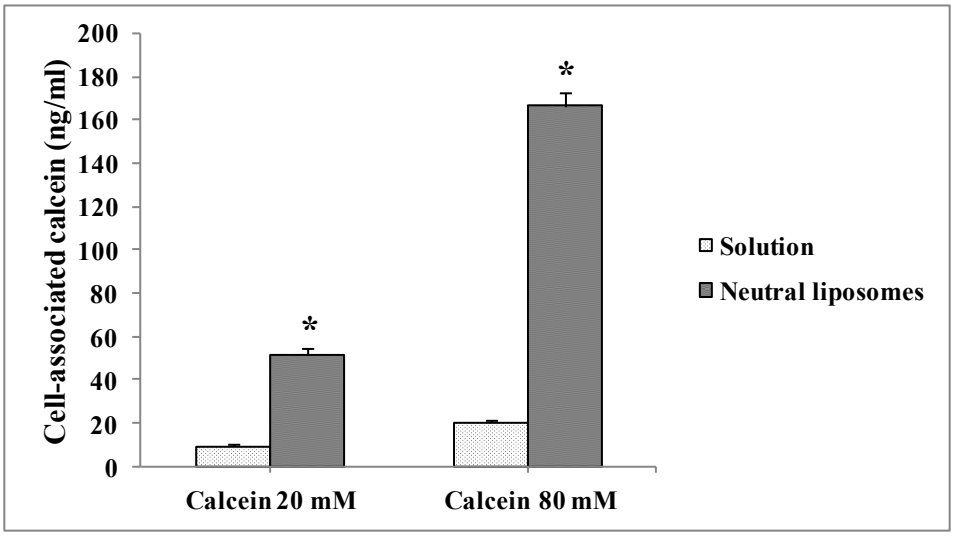

Figure 11: Cell-associated calcein from calcein solution and from neutral liposomes (at 20 and 80 mM of calcein) measured by the spectrofluorometric method after cell digestion. Data are shown as mean ± SEM (n = 3 batches). * $p < 0.05$, compared with solution

PEGylated liposomes

The positively charged PEGylated liposomes were used to verify the major mechanism of uptake of PEGylated liposomes into Caco-2 cells. Among the PEGylated liposomes studied, only this liposome type showed successful delivery of both calcein and calcein AM into Caco-2 cells (Figures 6 and 8). Fluorescence

histograms from flow cytometric measurement are shown in Figure 12. Mean fluorescence intensity calculated as % of the control is also summarized in Table 10.

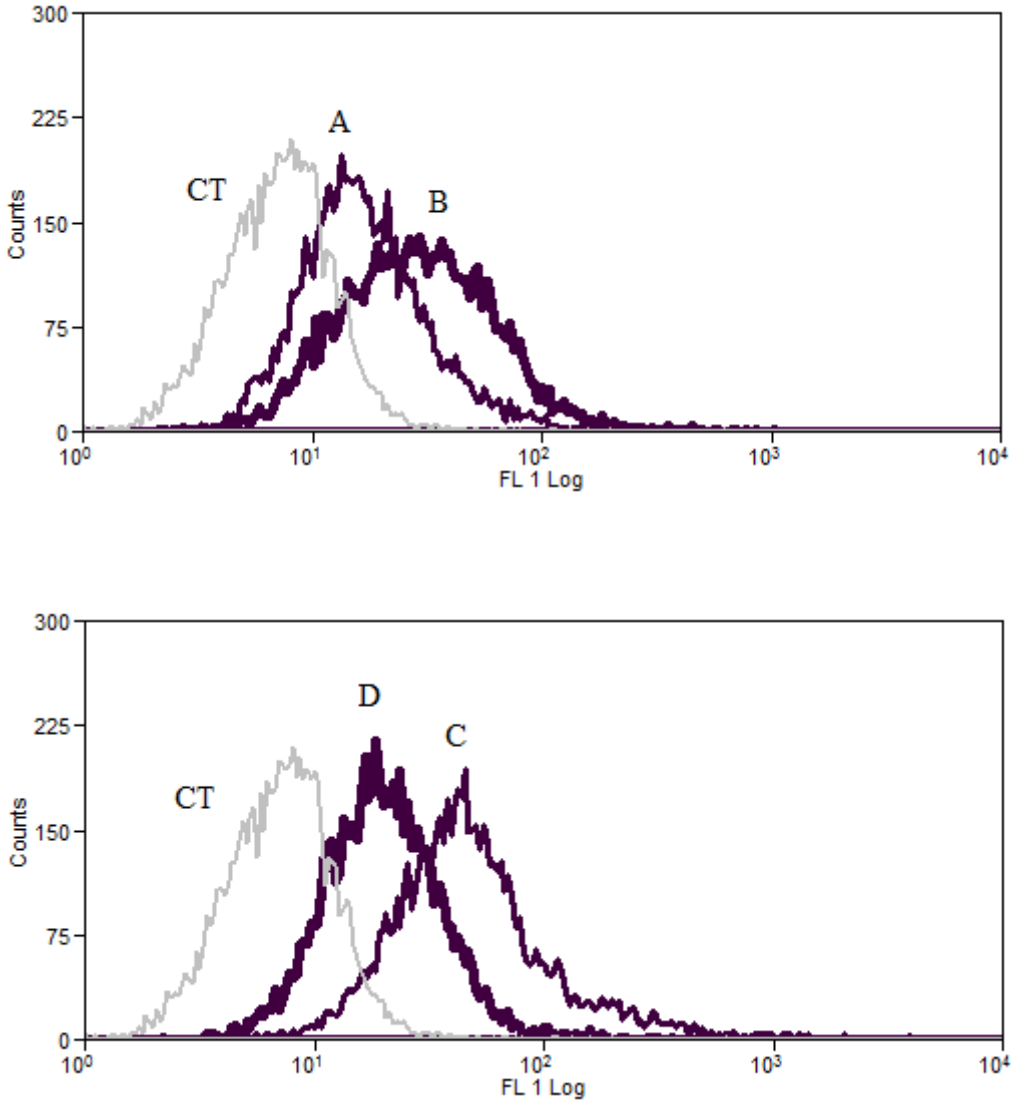

Figure 12: Fluorescence histograms from Caco-2 cells incubated with A) calcein solution at a dequenching concentration, B) PEG positive liposomes entrapping calcein at a dequenching concentration, C) calcein solution at a self-quenching concentration, and D) PEG positive liposomes entrapping calcein at a self-quenching concentration. Cells treated with serum-free DMEM were used as control (CT)

Table 10: Fluorescence intensity (% of control) of cell-associated calcein from the calcein solution and from the positively charged PEGylated liposomes measured by the flow cytometric method. Data are shown as % of control ± SEM (n = 3 batches).

	Fluorescence intensity (% of control)	
	Treatment with calcein at a dequenching concentration	Treatment with calcein at a self-quenching concentration
Solution	338.15 ± 11.26	950.42 ± 43.73
PEG positive liposomes	465.20 ± 10.58	346.81 ± 13.00

These results were in good agreement with those observed with the conventional liposomes. At a dequenching concentration of calcein (20 mM), the fluorescence intensity of calcein from the liposomes was markedly higher than that from the solution ($p < 0.05$). In contrast, at a self-quenching concentration of calcein (80 mM), the fluorescence intensity of calcein from the liposomes was significantly lower than that from the solution ($p < 0.05$). These results indicated that positively charged PEGylated liposomes were taken up by Caco-2 cells via endocytosis and not by fusion. Cell-associated calcein from calcein solution and from PEGylated positive liposomes (at 20 and 80 mM of calcein) after cell digestion are displayed in Figure 13. The similar results to those seen with the conventional liposomes were obtained, implying the same uptake mechanism. Though PEGylated liposomes seemed to be endocytosed by Caco-2 cells less efficiently than the conventional liposomes, they were still much more efficient in delivering their contents to the cells than the solution.

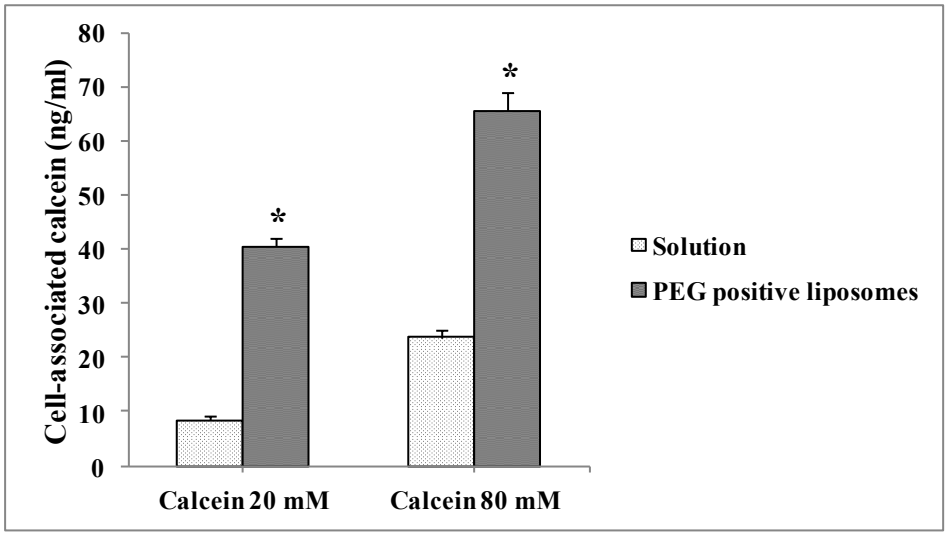

Figure 13: Cell-associated calcein from calcein solution and from PEGylated positive liposomes (at 20 and 80 mM of calcein) measured by the spectrofluorometric method after cell digestion. Data are shown as mean ± SEM (n = 3). * $p < 0.05$, compared with solution

As previously mentioned, the cellular uptake of liposomes is generally believed to be mediated by adsorption followed by endocytosis (Anderson et al., 2001; Chebbi et al., 2010; Miller et al., 1998; Ziello et al., 2010). Liposomes containing 1-Oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoglycerol and CH can deliver their contents into MDA-MB-231 human breast cancer cells via the endocytic pathway (Chebbi et al., 2010). The neutral and anionic small unilamellar liposomes were reported to be better internalized via endocytosis by the trophoblast cells when compared to the cationic liposomes (Bajoria et al., 1997). PEGylated liposomes have also been reported to be taken up by endocytosis (Chan et al., 2012; Miller et al., 1998). Moreover, most of liposomes are reported to be taken up into the cells by clathrin-mediated endocytosis (Ziello et al., 2010). The study by Un and co-workers (2012) elucidated that conventional liposomes composed of DOPC and CH were taken up by HeLa and HT-29 cells via clathrin-mediated endocytosis. The intracellular uptake of DOPC and CH was inhibited in the presence of chlorpromazine, a clathrin-mediated endocytosis inhibitor.

The results of this present study indicated that uptake of PEGylated phospholipid-based liposomes into Caco-2 cells also occurred via endocytosis.

CHAPTER V

CONCLUSIONS

In this study, Caco-2 cells were used as a model for intestinal epithelial cells to evaluate the feasibility of using PEGylated phospholipid-based liposomes for oral drug delivery. The emphasis was on drugs with low oral permeability, namely hydrophilic drugs and drugs that are substrates of P-glycoprotein (P-gp). Calcein and calcein AM were used as models for hydrophilic compounds and lipophilic P-gp substrates, respectively. Effects of surface charge and PEGylation on cellular liposome uptake were investigated and compared with conventional liposomes. The plausibility of using PEGylated liposomes to bypass the P-gp function was evaluated. The major mechanism in the uptake of PEGylated liposomes into Caco-2 cells was also verified.

The uptake studies indicated that the interaction of liposomes with Caco-2 cells was affected by both liposome surface charge and PEGylation. For calcein uptake, positively charged liposomes were the most efficient in the delivery of calcein into Caco-2 cells for both conventional and PEGylated liposomes. Neutral and negatively charged conventional liposomes displayed less calcein accumulation than the positively charged conventional liposomes. However, both liposome types still resulted in the higher calcein uptake than the solutions. The inclusion of PEG on the liposome surface resulted in a significant decrease in calcein uptake in all cases. The positively charged PEGylated liposomes, however, still delivered calcein to the cells in appreciable amounts compared to the solution. PEG neutral and negatively charged liposomes failed to deliver calcein to the cells.

The studies with calcein AM liposomes demonstrated that calcein AM from the conventional neutral, PEG neutral, and PEG positively charged liposomes were taken up by Caco-2 cells more efficiently than that from the calcein AM solution. Positively charged conventional liposomes failed to improve the delivery of calcein AM into Caco-2 cells. PEGylation seemed to have negligible effect on calcein AM uptake for both neutral and negatively charged liposomes. Interestingly, incorporation

of PEG into the positively charged liposomes could restore the advantage of liposomes on the calcein AM uptake.

Calcein also served as an aqueous phase liposome marker for investigating the major mechanism in the uptake of PEGylated liposomes by Caco-2 cells. The results from fluorescence dequenching technique comparing the fluorescence intensities from flow cytometric method and from spectrofluorometric method were consistent with endocytosis. Thus, the major mechanism in the uptake of PEGylated phospholipid-based liposomes was similar to that reported previously for conventional liposomes.

In summary, endocytosis was the major mechanism in the enhanced uptake of calcein and calcein AM from liposomes into Caco-2 cells. Liposome uptake by Caco-2 cells depended largely on liposome compositions, including surface charge and PEGylation, as well as the properties of the liposome contents. PEGylation tended to decrease liposome-cell interaction. Nevertheless, PEGylated phospholipid-based liposomes with the right composition were still efficiently taken up by Caco-2 cells. These PEGylated liposomes could deliver the hydrophilic compound calcein to the Caco-2 cells better than the solution and even than the conventional liposomes in some cases. Moreover, they were also efficient in bypassing the function of P-gp to deliver the hydrophobic P-gp substrate calcein AM to the cells. Therefore, by careful selection of their compositions, it would be possible to fabricate PEGylated liposome delivery systems for the lowly permeable hydrophilic substances and lipophilic P-gp substrates via the oral route. In order to realize these goals, however, some more research work should be carried out. These include the confirmation on the stability of PEGylated liposomes upon contact with GI fluids and the study of liposomal delivery to other GI cell types such as M-cells or via Peyer's patches. The study using other drug substances of interest should also be crucial. In vivo studies are necessary to evaluate the actual possibility of using PEGylated liposomes for oral delivery system.

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APPENDICES

APPENDIX A

Statistical test results for particle size of blank, calcein-loaded, and calcein AM-loaded liposomes

Statistical test results for particle size of blank liposomes

Dunnett T3

(I) Types of liposome	(J) Types of liposome	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Neutral	Positive	13.23333	2.18149	.097	-5.0003	31.4670
	Negative	28.97333*	3.73821	.017	8.2571	49.6896
	PEG neutral	7.63333	2.74752	.316	-6.8997	22.1663
	PEG positive	4.80000	2.14838	.552	-14.3913	23.9913
	PEG negative	6.16667	2.14113	.393	-13.2609	25.5942
Positive	Neutral	-13.23333	2.18149	.097	-31.4670	5.0003
	Negative	15.74000	3.10658	.147	-11.6022	43.0822
	PEG neutral	-5.60000	1.79598	.328	-19.9381	8.7381
	PEG positive	-8.43333*	.54058	.003	-11.6397	-5.2269
	PEG negative	-7.06667*	.51099	.007	-10.4821	-3.6512
Negative	Neutral	-28.97333*	3.73821	.017	-49.6896	-8.2571
	Positive	-15.74000	3.10658	.147	-43.0822	11.6022
	PEG neutral	-21.34000*	3.52716	.049	-42.5798	-.1002
	PEG positive	-24.17333	3.08343	.067	-52.2581	3.9114
	PEG negative	-22.80667	3.07838	.076	-51.0631	5.4498
PEG neutral	Neutral	-7.63333	2.74752	.316	-22.1663	6.8997
	Positive	5.60000	1.79598	.328	-8.7381	19.9381
	Negative	21.34000*	3.52716	.049	.1002	42.5798
	PEG positive	-2.83333	1.75563	.765	-18.2294	12.5627
	PEG negative	-1.46667	1.74674	.984	-17.1402	14.2069
PEG positive	Neutral	-4.80000	2.14838	.552	-23.9913	14.3913
	Positive	8.43333*	.54058	.003	5.2269	11.6397
	Negative	24.17333	3.08343	.067	-3.9114	52.2581
	PEG neutral	2.83333	1.75563	.765	-12.5627	18.2294
	PEG negative	1.36667	.34319	.126	-.4785	3.2118

(I) Types of liposome	(J) Types of liposome	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PEG negative	Neutral	-6.16667	2.14113	.393	-25.5942	13.2609
	Positive	7.06667*	.51099	.007	3.6512	10.4821
	Negative	22.80667	3.07838	.076	-5.4498	51.0631
	PEG neutral	1.46667	1.74674	.984	-14.2069	17.1402
	PEG positive	-1.36667	.34319	.126	-3.2118	.4785

* The mean difference is significant at the 0.05 level.

Statistical test results for particle size of calcein-loaded liposomes

Tukey HSD

(I) Types of liposome	(J) Types of liposome	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Neutral	Positive	5.34667	1.59879	.051	-.0235	10.7169
	Negative	14.25000*	1.59879	.000	8.8798	19.6202
	PEG neutral	-.08000	1.59879	1.000	-5.4502	5.2902
	PEG positive	-2.24333	1.59879	.725	-7.6135	3.1269
	PEG negative	1.16000	1.59879	.975	-4.2102	6.5302
Positive	Neutral	-5.34667	1.59879	.051	-10.7169	.0235
	Negative	8.90333*	1.59879	.001	3.5331	14.2735
	PEG neutral	-5.42667*	1.59879	.047	-10.7969	-.0565
	PEG positive	-7.59000*	1.59879	.005	-12.9602	-2.2198
	PEG negative	-4.18667	1.59879	.166	-9.5569	1.1835
Negative	Neutral	-14.25000*	1.59879	.000	-19.6202	-8.8798
	Positive	-8.90333*	1.59879	.001	-14.2735	-3.5331
	PEG neutral	-14.33000*	1.59879	.000	-19.7002	-8.9598
	PEG positive	-16.49333*	1.59879	.000	-21.8635	-11.1231
	PEG negative	-13.09000*	1.59879	.000	-18.4602	-7.7198

(I) Types of liposome	(J) Types of liposome	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PEG neutral	Neutral	.08000	1.59879	1.000	-5.2902	5.4502
	Positive	5.42667*	1.59879	.047	.0565	10.7969
	Negative	14.33000*	1.59879	.000	8.9598	19.7002
	PEG positive	-2.16333	1.59879	.752	-7.5335	3.2069
	PEG negative	1.24000	1.59879	.967	-4.1302	6.6102
PEG positive	Neutral	2.24333	1.59879	.725	-3.1269	7.6135
	Positive	7.59000*	1.59879	.005	2.2198	12.9602
	Negative	16.49333*	1.59879	.000	11.1231	21.8635
	PEG neutral	2.16333	1.59879	.752	-3.2069	7.5335
	PEG negative	3.40333	1.59879	.335	-1.9669	8.7735
PEG negative	Neutral	-1.16000	1.59879	.975	-6.5302	4.2102
	Positive	4.18667	1.59879	.166	-1.1835	9.5569
	Negative	13.09000*	1.59879	.000	7.7198	18.4602
	PEG neutral	-1.24000	1.59879	.967	-6.6102	4.1302
	PEG positive	-3.40333	1.59879	.335	-8.7735	1.9669

* The mean difference is significant at the 0.05 level.

Statistical test results for particle size of calcein AM-loaded liposomes

Tukey HSD

(I) Types of liposome	(J) Types of liposome	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Neutral	Positive	7.66667*	1.68142	.007	2.0189	13.3144
	Negative	13.80000*	1.68142	.000	8.1522	19.4478
	PEG neutral	.37667	1.68142	1.000	-5.2711	6.0244
	PEG positive	5.79667*	1.68142	.043	.1489	11.4444
	PEG negative	9.43333*	1.68142	.001	3.7856	15.0811

(I) Types of liposome	(J) Types of liposome	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Positive	Neutral	-7.66667*	1.68142	.007	-13.3144	-2.0189
	Negative	6.13333*	1.68142	.031	.4856	11.7811
	PEG neutral	-7.29000*	1.68142	.010	-12.9378	-1.6422
	PEG positive	-1.87000	1.68142	.867	-7.5178	3.7778
	PEG negative	1.76667	1.68142	.891	-3.8811	7.4144
Negative	Neutral	-13.80000*	1.68142	.000	-19.4478	-8.1522
	Positive	-6.13333*	1.68142	.031	-11.7811	-.4856
	PEG neutral	-13.42333*	1.68142	.000	-19.0711	-7.7756
	PEG positive	-8.00333*	1.68142	.005	-13.6511	-2.3556
	PEG negative	-4.36667	1.68142	.171	-10.0144	1.2811
PEG neutral	Neutral	-.37667	1.68142	1.000	-6.0244	5.2711
	Positive	7.29000*	1.68142	.010	1.6422	12.9378
	Negative	13.42333*	1.68142	.000	7.7756	19.0711
	PEG positive	5.42000	1.68142	.063	-.2278	11.0678
	PEG negative	9.05667*	1.68142	.002	3.4089	14.7044
PEG positive	Neutral	-5.79667*	1.68142	.043	-11.4444	-.1489
	Positive	1.87000	1.68142	.867	-3.7778	7.5178
	Negative	8.00333*	1.68142	.005	2.3556	13.6511
	PEG neutral	-5.42000	1.68142	.063	-11.0678	.2278
	PEG negative	3.63667	1.68142	.320	-2.0111	9.2844
PEG negative	Neutral	-9.43333*	1.68142	.001	-15.0811	-3.7856
	Positive	-1.76667	1.68142	.891	-7.4144	3.8811
	Negative	4.36667	1.68142	.171	-1.2811	10.0144
	PEG neutral	-9.05667*	1.68142	.002	-14.7044	-3.4089
	PEG positive	-3.63667	1.68142	.320	-9.2844	2.0111

* The mean difference is significant at the 0.05 level.

APPENDIX B

Molecular structures of calcein, calcein AM,

cyclosporin A, and indomethacin

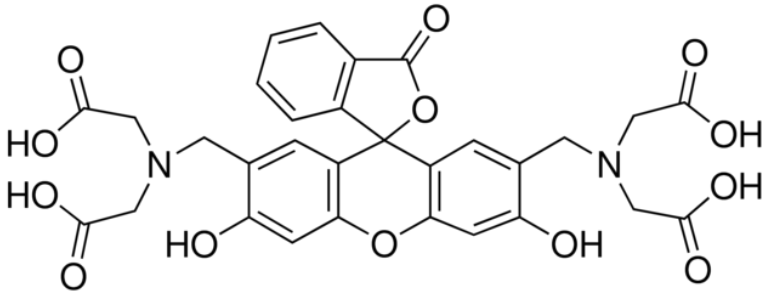
(Sigma-Aldrich, 2013)

Calcein (Sigma-Addrich, 2013)

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

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

Structure:



(From Sigma-Aldrich, 2013)

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

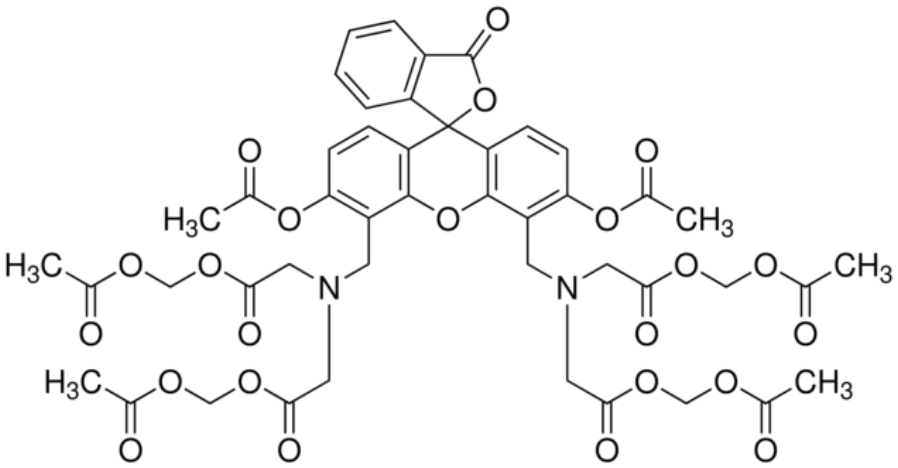
Calcein AM (Sigma-Addrich, 2013)

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

Calcein acetoxymethyl ester

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

Structure:



(From Sigma-Aldrich, 2013)

Molecular weight: 994.86

Solubility: soluble in DMSO

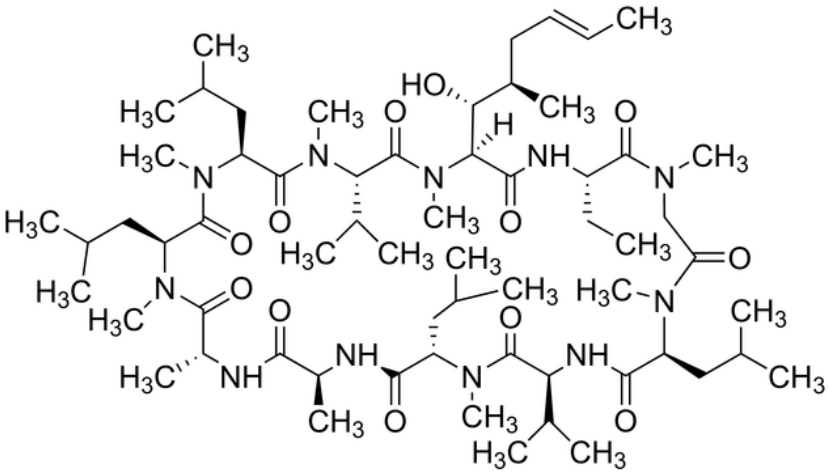
Storage temperature: -20 °C

Cyclosporin A (Sigma-Addrich, 2013)

Synonym: Cyclosporine, Antibiotic S 7481F1

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

Structure:



(From Sigma-Aldrich, 2013)

Molecular weight: 1202.61

Solubility: soluble in dichloromethane, ethanol, DMSO, chloroform

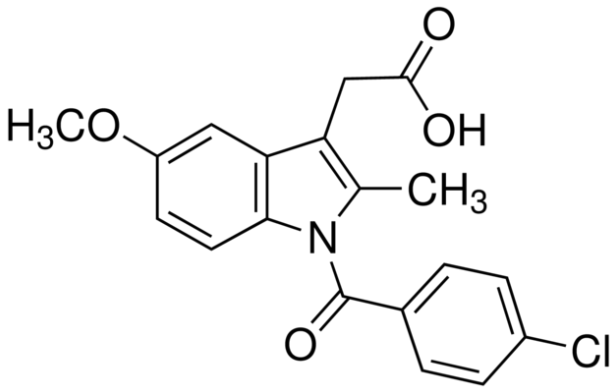
Storage temperature: 2-8 °C

Indomethacin (Sigma-Addrich, 2013)

Synonym: 1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid

Empirical: $C_{19}H_{16}ClNO_4$

Structure:



(From Sigma-Aldrich, 2013)

Molecular weight: 357.79

Solubility: soluble in ethanol, DMSO

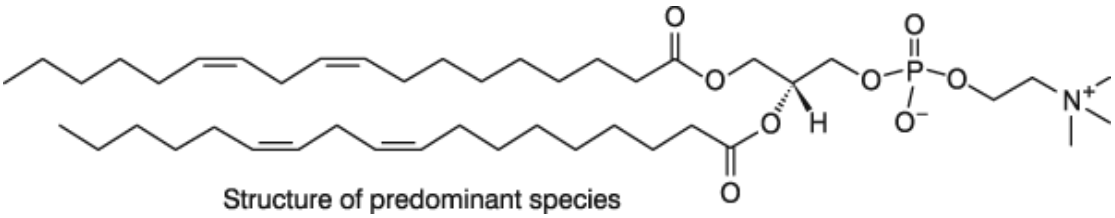
Storage temperature: -20 °C

APPENDIX C

Molecular structures of PC, CH, PEG2000-PE, SA, and DCP

(Avanti Polar Lipids; Sigma-Aldrich, 2013)

Molecular structure of phosphatidylcholine



(From Avanti Polar Lipids, 2013)

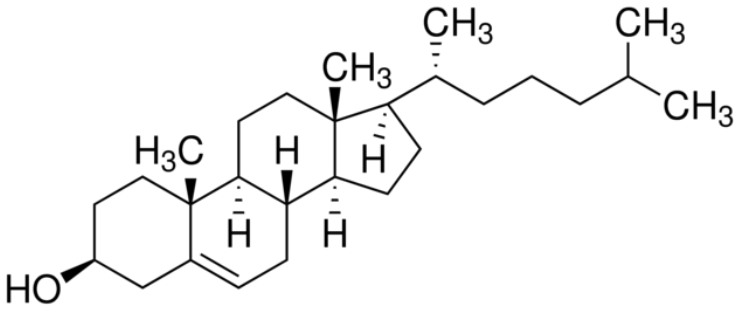
Cholesterol

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

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

Molecular weight: 386.65

Structure:



(From Sigma-Aldrich, 2013)

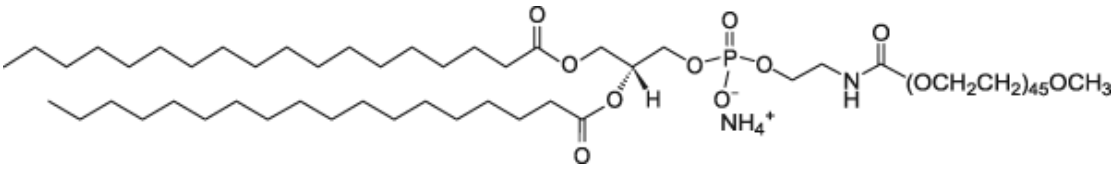
PEG2000-PE

Synonym: DSPE-mPEG(2000)

Empirical: $C_{133}H_{267}N_2O_{55}P$

Molecular weight: 2805.497

Structure:



(From Sigma-Aldrich, 2013)

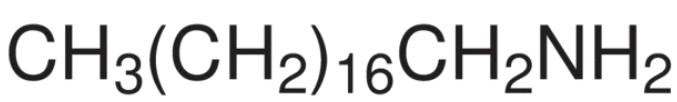
Stearylamine (SA)

Synonym: 1-Aminooctadecane, octadecylamine

Empirical: $C_{18}H_{39}N$

Molecular weight: 269.51

Structure:



(From Sigma-Aldrich, 2013)

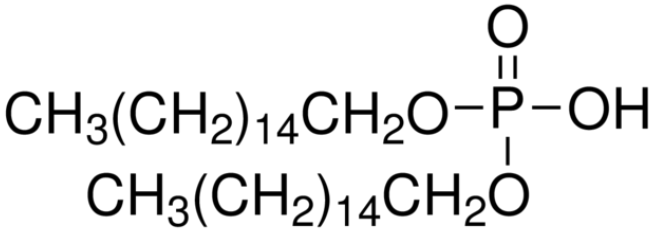
Dicetylphosphate (DCP)

Synonym: dihexadecyl phosphate, DHP

Empirical: $C_{32}H_{67}O_4P$

Molecular weight: 546.85

Structure:



(From Sigma-Aldrich, 2013)

APPENDIX D

P-gp and MRP2 functions in Caco-2 monolayers

P-gp function

Cell passage numbers	AEF
37	2.69
38	2.70
40	2.64
65	2.99
66	2.84
Mean ± SD	2.77 ± 0.14

MRP2 function

Cell passage numbers	AEF
37	1.69
65	1.64
66	1.47
Mean ± SD	1.60 ± 0.12

APPENDIX E

Uptake profiles of calcein and calcein AM from solution and liposomes

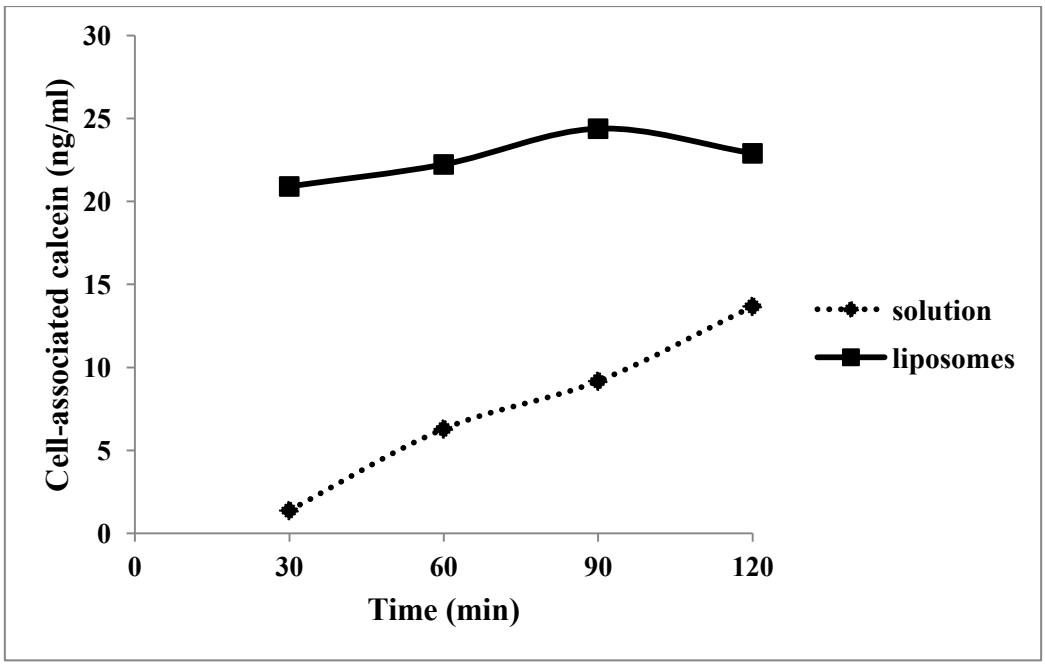


Figure 14: Calcein uptake profiles from calcein-loaded neutral liposomes with the lipid concentration of 0.35 mg/ml and calcein solutions at the corresponding calcein concentration. Data are shown as an average of 3 wells in 1 passage of cells.

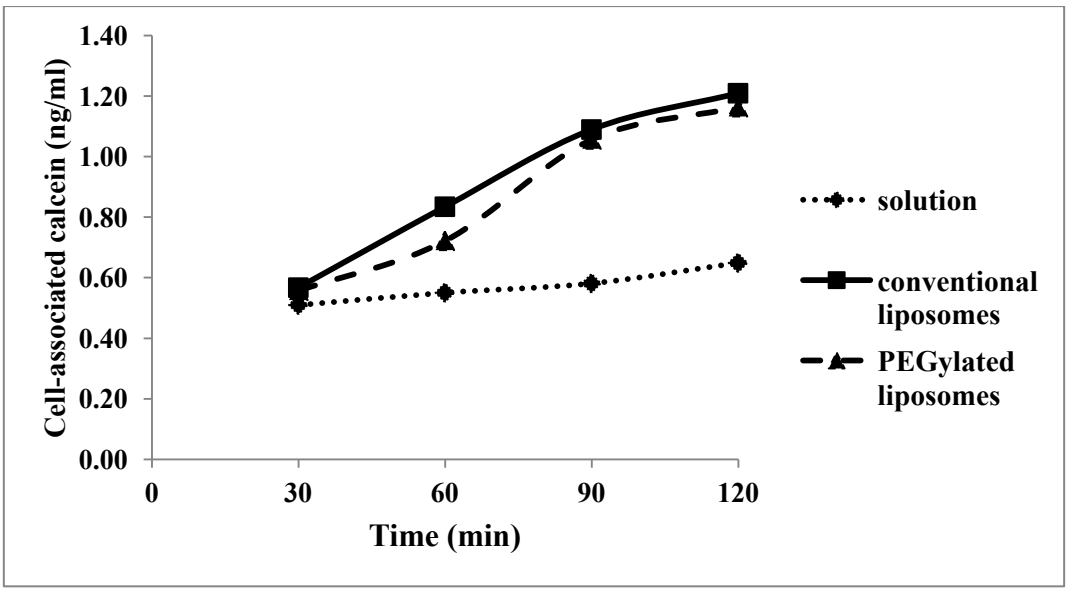


Figure 15: Calcein AM uptake profiles from calcein AM-loaded neutral liposomes, calcein AM-loaded PEG neutral liposomes with the lipid concentration of 0.35 mg/ml and calcein AM solution. Data are shown as an average of 3 wells in 1 passage of cells.

VITA

Miss Jitkasem Meewan was born on October 26, 1983, in Udon Thani, Thailand. She graduated with a Bachelor of Science Degree in Pharmacy (second-class honors) from the Faculty of Pharmacy, Chiang Mai University in 2006. She worked at Boots Retail, Bangkok, Thailand as a pharmacist for two years before enrolling in the Master of Science Program in Pharmaceutical Technology (International) at Chulalongkorn University in Bangkok, Thailand.