CHAPTER III

MATERIALS AND METHODS

Materials

1. Advanced Dulbecco's Modified Eagle's Medium (GibcoBRL, Lot no. 1187889)
2. Calcein (Sigma, USA, Lot no. 20K0575)
3. Calcium chloride (Merck, Germany, Lot no. TA795482)
4. Chloroform, HPLC grade (Labscan, Irland, Lot no. 03100062)
5. Cholesterol (Sigma, USA, Lot no. 58H5234)
6. D-Glucose monohydrate (Riedel-de Waen, Germany, Lot no. 1R20334)
7. Dicetylphosphate (Sigma, USA, Lot no. 10K1593)
8. Dimetyl sulphoxide (DMSO) (Sigma, USA, Lot no. 24K2300)
9. Dulbecco's Modified Eagle's Medium (GibcoBRL, USA, Lot no. 1188491)
10. EDTA ([Ethylenedinitrilo]tetraacetic acid) (Sigma, USA, Lot no. 85H00085)
11. Fetal bovine serum (Biochrom AG, USA, Lot no. 0830G, 256FF, 992FF and 1055FF)
12. Hank's balanced salt solution (Sigma, USA, Lot no. 103K83021)
13. HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, USA, Lot no. 79F-5602)
14. L-Glutamine (GibcoBRL, USA, Lot no. 1160728, 1167436)
15. Magnesium chloride (Merck, Germany, Lot no. A776332)
16. MES (2-[N-Marpholino]ethanesulfonic acid) (Sigma, USA, Lot no. M8250)
17. Non essential amino acid (Sigma, USA, Lot no. 44K2415)
18. Penicillin-Streptomycin (GibcoBRL, USA, Lot no. 1211219)
19. Phenol red (Fluka, Switzerland, Lot no. G00784)
20. Phosphatidylcholine (Phospholipon®90 Nattermann Phospholipid GmbH, Cologne, Germany, Lot no. 770991)
21. Potassium chloride (Sigma, USA, Lot no. 34H0302)

22. Potassium dihydrogen phosphate (Merck, Germany, Lot no. A255673)
23. Potassium hydroxide (Merck, Germany, Lot no. 482233)
24. Rhodamine 123 (Sigma, USA, Lot no. 014K3689)
25. Sephadex G-50 (Sigma, USA, Lot no. 115H0876)
26. Sodium bicarbonate (Merck, Germany, Lot no. K00415629)
27. Sodium chloride (Merck, Germany, Lot no. K01658204)
28. Sodium hydroxide (Merck, Germany, Lot no. B870498625)
29. Sodium phosphate dibasic anhydrous (Fluka, Switzerland, Lot no. R1376)
30. Stearylamine (Sigma, USA, Lot no. 45H3435)
31. Theophylline (Supplied by B.L Hua, Thailand, Lot no. S99123)
32. 1% Triton X-100 (Fluka, Switzerland, Lot no. 279624)
33. Trypan blue (Sigma, USA, Lot no. 101K3681)
34. Trypsin (Sigma, USA, Lot no. 064K7696)
35. α-Tocopherol (Approx. 95%, Sigma, USA, Lot no. 53H0444)
36. ±Verapamil hydrochoride (Sigma, USA, Lot no. 062K0325)

Equipment

1. Analytical balances (GMPH, Satorius, Germany; UMT2, Mettler Toledo, Switzerland)
2. Autoclave (Hirayama, Japan)
3. Counting chamber (BOECO, Germany)
4. FACS Calibur (Becton Dickinson, USA)
5. Flex-column (Kontes, USA)
6. Fluorescence microplate reader (VICTOR3, Perkin Elmer, USA)
7. Fluorescence microscope (BX-FLA, Olympus, Japan)
8. Hand-held extruder (LiposoFast^{TM}, AVESTIN, Canada)
9. Hot air oven (MEMMERT, Germany)
10. Humidified carbon dioxide incubator (Forma Scientific, USA)
11. Laminar air flow hood (BV2225, ISSCO, Thailand)
12. Light microscope (Leica ATC2000, Olympus, Japan)

13. Milicell®-ERS potentiometer (Millipore, USA)
14. Multiwell plates (Nunc, Denmark and Corning, USA)
15. pH meter (Beckman, USA)
16. Polycarbonate membranes (100 and 200 nm) (Avestin, Canada)
17. Refrigerated centrifuge (ALC4237R, ALC International, Italy)
18. Rotary evaporator (RE120, Buchi, Switzerland)
19. Shaking incubator (MTS 4, Kika, Germany)
20. Sonication (Elma, Germany)
21. Spectrofluorometer (FP777, Jasco Corporation, Japan)
22. Sterilization filtration membranes (cellulose acetate membrane, 0.22 μm) (Corning, USA and Roseville, Michigan)
23. Transwell® insert (Corning, USA)
24. Tissue culture flasks (Nunc, Denmark and Corning, USA)
25. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)
26. Vortex mixer (G-560E, Scientific Industries, USA)

Methods

1. Selection of medium for cell growth

Invitrogen Corporation has introduced a new advanced medium with additional amino acids and sodium pyruvate, ethanolamine, glutathione, ascorbic acid phosphate, insulin, transferrin, Albumax®, and trace elements. According to the manufacturer, this new medium, Advanced Dulbecco's Modified Eagle's Medium (Advanced DMEM), could enable users to reduce fetal bovine serum (FBS) supplementation by 50-90 % with no change in cell growth, morphology, or function of cell. The purpose of this experiment was to test whether this medium could satisfactorily replace the conventional Dulbecco's Modified Eagle's Medium (DMEM) for Caco-2 cell propagation with a reduced cost.

In all experiments, Caco-2 cells were cultivated under standard conditions in a humidified CO_2 incubator with 90% relative humidity, at 37 °C and 5% CO_2. Caco-2 cells grown in a culture flask with standard DMEM was trypsinized, washed, and resuspended in each of the media to be tested. Two milliliters of the cell suspension (6×10^4 cells per ml) was seeded into each well of 6-well culture plates. Cells were allowed to grow in either standard DMEM with 10% FBS or in Advanced DMEM supplemented with 2% or 4% FBS (Table 3). The medium was replaced every 48 hours. At various days after seeding, cells in each well were washed with 2 ml PBS and trypsinized using 0.25% trypsin in 1mM EDTA solution. Cells were resuspended in fresh medium. The dispersed Caco-2 cells were stained with 0.4% trypan blue dye and counted on a hemacytometer. Data were collected throughout 14 days. Growth curves were constructed between cell concentration (cells per ml) and culture time on a semilog scale. The lag time and the population-doubling time (PDT) were determined from the growth curve.

Table 3: Compositions of the three media tested

Major component / Supplement	DMEM	Advanced DMEM	Advanced DMEM
Fetal bovine serum	10%	2%	4%
MEM-Non-essential amino acid	1%	1%	1%
Sodium pyruvate	1%	1%	1%
L-glutamine	1%	1%	1%
Penicillin-Streptomycin	1%	0.2%	0.4%

2. Maintenance of Caco-2 cells

Caco-2 cells were grown in the medium with supplements selected from Experiment 1. Caco-2 cells were routinely seeded at initial density of 5-6 x 10^5 cells per 15 ml in 75-cm^2 tissue culture flasks. Cells were cultured in a humidified CO_2 incubator at 37 °C and 5% CO_2. The cells were subcultured at no more than 70% confluence (approximately 3-5 days after seeding) with passage ratio of 1:2 (v/v). The cell monolayer was washed with 5 ml of phosphate buffered saline (PBS) and detached from the culture flask by incubating with 0.25% trypsin in 1mM EDTA solution (2 ml/75 cm^2) for 2-3 minutes at room temperature. Ten milliliters of the medium was added to stop the action of trypsin. The cell suspension was triturated up and down with a glass pipette to disperse the cells. The dispersed cell suspension was transferred to a centrifuge tube and centrifuged at 1,200 rpm for 4 minutes (ALC4237R, ALC International, Italy). The supernatant was discarded and the pellet was resuspended in the medium. Cells were counted and seeded into new flasks. Cell suspension was seeded at 5-6 x 10^5 cells per 15 ml in each 75-cm^2 culture flask.

3. Characterization of Caco-2 monolayers

In order to use Caco-2 monolayers as a tool to study drug uptake and transport, the monolayers should be standardized in terms of integrity of the monolayer as well as functionality of the cells. Caco-2 monolayers ought to have high transepithelial electrical resistance (TEER) values after confluence, be good barrier to hydrophilic substances,

have high permeation to small lipophilic substances, and express efflux transporters of interest.

3.1 Characterization of integrity of Caco-2 monolayers: the paracellular pathway

To test whether liposomes would facilitate drug uptake into the cell and through the monolayer, tight monolayers that restricted drug passage through the paracellular pathway were required. Transepithelial electrical resistance and permeability of a hydrophilic marker, phenol red, were used to confirm integrity of Caco-2 monolayers.

3.1.1 Transepithelial electrical resistance measurements

The purpose of this experiment was to characterize formation of tight junctions. Caco-2 monolayers with good tight junction integrity should display transepithelial electrical resistance (TEER) of 300-1,400 $\Omega \cdot cm^2$ (Hidalgo, 2001; Borchard et al., 1996). Transwell® inserts (see picture in Appendix I) were measured for a baseline TEER value with a Millicell®-ERS potentiometer (Millipore, USA) before the cells were seeded onto their polycarbonate membranes (3 μm in pore size, 24 mm in diameter) at a concentration of 2.83 x 10^5 cells per 4.71 cm^2. Caco-2 cells used in this study were between the passage number of 50 and 80 as recommended (Balimane, Han, and Chong, 2006). Cells were grown under the standard conditions. The medium was replaced every 48 hours for 21 days. TEER was measured every 2 days after the monolayer had completely reached confluence. TEER values of Caco-2 monolayers were determined as follows:

$$TEER_{monolayer} = (TEER_{total} - TEER_{membrane}) \times A$$

where $TEER_{monolayer}$ = TEER of Caco-2 monolayer at the specified time ($\Omega \cdot cm^2$)

$TEER_{total}$ = Total TEER measured at the specified time

$TEER_{membrane}$ = TEER of the polycarbonate membrane measured before cell seeding

A = area of Transwell® insert (cm^2)

3.1.2 Permeability of phenol red

Tight junction integrity may be assessed by measuring transport of hydrophilic substances such as Lucifer yellow and phenol red. From the literature, Caco-2 monolayers were considered tight when permeability of Lucifer yellow was less than 1% (Prueksaritanont et al., 1998) and that of phenol red was less than 5% (Martel, Monteiro, and Lemos, 2003) per hour.

For transport study, the Caco-2 monolayer on the insert was rinsed with the transport medium. Caco-2 monolayers were then pre-incubated with the apical buffer, pH 6.5, on the apical side and the basolateral buffer, pH 7.4, on the basolateral side at 37 °C for 30 min. The complete compositions of these buffers are displayed in Appendix II. The apical buffer was replaced with the apical buffer that contained 500 μM of phenol red. The basolateral side was replaced with fresh basolateral buffer. Transwell® plate was kept shaken at 75 rpm/min on an orbital shaker (Kika, Germany). The volume of the apical buffer was 1.5 ml and that of the basolateral buffer was 2.6 ml. Transport was studied in the apical to basolateral direction, at 60 min intervals. The extent of phenol red transport was quantified by UV-spetrophotometric method at 430 nm.

3.2 Characterization of permeability of Caco-2 monolayers: the transcellular pathway

Rapidly and completely absorbed drugs are generally small lipophilic substances that can distribute readily into the cell membrane. Several lipophilic substances such as danazol, ketoconazol, and theophylline have been used to study drug transport through the transcellular pathway of Caco-2 monolayers (Ingels et al., 2002; Ingels et al., 2004). In this experiment, theophylline was used as a marker molecule to assess whether the transcellular permeability of the developed Caco-2 monolayers were comparable to those in the literature.

Transport study was performed as described under 3.1.2. For drug transport in the direction of apical to basolateral (A-to-B), the apical buffer was replaced with the apical buffer containing 200 μM of theophylline (instead of phenol red). To maintain sink

conditions during the experiment, Transwell® inserts were transferred to new wells containing fresh buffer every 15 min. The basolateral buffer was analyzed for theophylline by UV-spectrophotometric method at 272 nm. For basolateral to apical (B-to-A) transport, the basolateral buffer was replaced with the basolateral buffer containing 200 μM of theophylline. Samples were taken from the apical side every 10 min with replacement of an equal volume of fresh buffer.

Transport of the drug was monitored for a period of 90 or 60 min for A-to-B or B-to-A direction, respectively. The apparent permeability coefficient (P_{app}) of theophylline was calculated as follows:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_0 A}$$

where P_{app} = apparent permeability coefficient (cm/sec)

dQ/dt = the amount of theophylline appearing in the receiver (or the donor) compartment as a function of time (nmol/sec)

A = the surface area across which the transport occurred (cm^2)

C_0 = the initial concentration in the donor compartment (μM) (assuming sink conditions)

3.3 Characterization of P-glycoprotein expression of Caco-2 monolayer

3.3.1 Expression of efflux transporters

This experiment was to determine whether Caco-2 monolayers cultivated under the conditions used could express any efflux transporters. Propranolol and rhodamine 123, which are known substrates of efflux pumps, were used as marker molecules. The efflux ratio, which compares the permeability in the B-to-A direction with that in the A-to-B direction, was determined and compared with the values reported in the literature.

With propranolol, the study was the same as that described for theophylline under Section 3.2. The concentration of propranolol was 200 μM. For

A-to-B permeability, the insert was removed to a new well every 10 min. For B-to-A permeability, samples (750 µl) were taken every 10 min. The study was continued over a period of 60 min. The amount of propranolol transported was quantified by UV-spectrophotometric method at 220 nm.

With rhodamine 123, the concentration used was 20 µM. Due to the slower transport of rhodamine 123 compared to that of propranolol, aliquots were taken instead of the insert being removed to a new well for both A-to-B and B-to-A directions. For the A-to-B transport, samples (1 ml) were taken from the basolateral compartment every 10 min. For the B-to-A transport, aliquots (750 µl) were taken from the apical compartment every 3 min. Rhodamine 123 was quantified by spectrofluorometric method with the excitation wavelength of 485 nm and the emission wavelength of 530 nm. Transport was monitors for 90 and 21 min for the A-to-B and the B-to-A directions, respectively. The apparent permeability coefficient in the A-to-B direction ($P_{app, AB}$) and the apparent permeability coefficient in the B-to-A direction ($P_{app, BA}$) were calculated. The efflux ratio was determined according to the following equation:

$$\text{Efflux ratio} = P_{app, BA} / P_{app, AB}$$

3.3.2 P-glycoprotein expression of Caco-2 monolayers

The purpose of this experiment was to confirm that the efflux transporter expressed in Caco-2 monolayers involved P-glycoprotein. Verapamil was used as a specific inhibitor of P-glycoprotein. The efflux ratios of rhodamine 123 in the absence and in the presence of verapamil were determined and compared. Rhodamine 123 concentration was 20 µM. Verapamil was used at 100 µM. In the experiment with verapamil, Caco-2 monolayer was pre-incubated with 100 µM verapamil in transport buffer at 37 °C for 30 min. The medium was then changed to transport buffer containing both verapamil and rhodamine 123 at 100 µM and 20 µM, respectively. The experiment was carried out as described under 3.3.1.

4. Effect of liposomes on accumulation of calcein in Caco-2 cells

The purpose of this experiment was to determine whether liposomes could increase accumulation of hydrophilic substances in the cells. The possible mechanism by which liposomes enhanced accumulation of hydrophilic substances in the cells was also studied. Calcein was used as a marker for hydrophilic molecules. It is a water-soluble fluorescent dye that has self-quenching at high concentrations. It has been used widely in liposome research. The fluorescence of calcein is less pH-dependent than other derivatives of fluorescein (New, 1997).

4.1 Preparation of calcein-loaded liposomes

Calcein-loaded liposomes were prepared by film-hydration method at total lipid concentration of 50 mg/ml. The composition of liposomes comprised phosphatidylcholine (PC) and cholesterol (CH) at a molar ratio of 7:3 for neutral liposomes. For positively and negatively charged liposomes, the compositions were PC, CH and either stearylamine (SA) or dicetylphosphate (DCP) at a molar ratio of 6:3:1, respectively. Alpa-tocopherol (0.1 mol%) was used as an antioxidant in all preparations. Briefly, the lipids were dissolved in chloroform in a round-shaped flask. The solution was evaporated using a rotary evaporator to leave a thin lipid film. The lipid film was kept under vacuum for at least 2 hours to eradicate traces of the organic solvent. The lipid film was hydrated by the aqueous phase (calcein 80 mM in 0.3 N NaOH). The liposomal vesicles formed spontaneously after lipid hydration. The resultant liposomal suspension was examined under a light microscope as a routine check of quality of the preparation.

The liposomal suspension was then extruded through two-stacked 200 nm polycarbonate membranes with a hand-held extruder for 19 cycles. The extrusion process was repeated through another set of 100 nm membranes to reduce liposome size to approximately 100 nm. Non-encapsulated calcein was separated from calcein-loaded liposomes by gel filtration using Sephadex G-50 (0.1 ml suspension loaded on a 1x 21 cm gel bed). The eluting fluid was PBS. After gel filtration, encapsulated calcein in liposomes was assayed as described under Section 4.2 that follows. The liposomal

suspension was kept in a refrigerator until use. The calcein-loaded liposomal suspension was used within the same day of preparation to avoid liposome leakage and bleaching of the fluorescent marker.

4.2 Determination of calcein encapsulation efficiency

The calcein-loaded liposomal suspension was diluted until the concentration of the lipid was 0.15 mg/ml with PBS. The liposomal suspension was allowed to dissolve in 1 % Triton X-100 for 45 min. The solution was diluted until concentration was within the desired range. Calcein was quantified by spectrofluorometric method at the excitation wavelength of 490 nm and emission wavelength of 510 nm.

4.3 Effects of time after seeding and calcein concentration on accumulation of calcein in Caco-2 cells

Cellular uptake is dependent on several factors such as total number of cells and concentration of substance to be taken up. The purpose of this experiment was to compare the uptake of calcein from solution and from liposomes at various times after cell seeding and at various concentrations of calcein. The time after seeding at which the maximum difference in uptake was seen would be used for further experiments, where the mechanism of liposome-enhanced uptake would be explored in more detail.

Caco-2 cells were seeded at a concentration of 25,000 cells/ml in 12-well culture plates with a final volume of 1 ml per well. Cells were allowed to grow for 3 days, 5 days, and 7 days under the normal culture conditions. Under these conditions, the culture reached confluence within 7 days. At 3 and 5 days, cells were in pre-confluence stage. At the specified intervals, cells were washed and pre-incubated at 37 °C for 30 min with serum-free medium. The medium was then replaced with either PC liposomes or calcein solution at concentrations of calcein equivalent to 50 μM, 75 μM, and 100 μM in 1 ml serum-free medium. The cells were further incubated at 37 °C for 2 hours. After that, the medium was removed from each well and cells were washed 4 times with ice-cold PBS. Cells were digested with 1 ml of 1% Triton X-100 at 37 °C for 2 hours.

Measurement of total calcein associated with the cells in each sample was done by spectrofluorometric method (excitation wavelength = 490 nm, emission wavelength = 510 nm). The standard curve was prepared in the medium with the similar composition to that used in the experiment, i.e. Triton X-100 was also included. Digested untreated cells were used for background correction. The preliminary study indicated that presence of cells did not alter calcein fluorencence under the conditions used.

4.4 Concentration dependence of calcein uptake by Caco-2 cells

Endocytosis, which was the expected mechanism for liposome uptake by cells in this study, is a carrier-mediated process. Saturation of the process at high concentration of liposomes was anticipated. The purpose of this experiment was to study whether calcein uptake by Caco-2 cells from solution and from calcein-loaded liposomes displayed any saturation. Caco-2 cells were seeded at a concentration of 25,000 cells/ml (final volume 1 ml per well) in 12-well culture plates. The culture was allowed to grow for the period selected from Section 4.3. The experiment was carried out as described under Section 4.3. Caco-2 cells were incubated for 2 hr with either calcein solution or calcein-loaded liposomes containing an equivalent amount to make 20 μM-140 μM calcein in serum-free medium with a final volume of 1 ml. Amounts of calcein taken up by cells were assayed and compared.

4.5 Mechanism of cellular uptake of calcein-loaded liposomes

Endocytosis is an active-transport process. The process is dependent on temperature since the functions of carrier proteins and enzymes involved in ATP breakdown are usually modulated by cell membrane fluidity (Hwang and Kim, 2005). The purpose of this experiment was to study the effect of temperature on the extent of calcein uptake from solution and from calcein-loaded liposomes. In addition, for hydrophilic molecules, the molecules taken up by endocytosis are mostly confined in the endosomes or lysosomes until exocytosis occurs. Thus, with self-quenching fluorophores

such as calcein, analysis of associated calcein with flow cytometry is useful in discriminating liposome-cell interaction by fusion from that by endocytosis.

4.5.1 Effect of temperature on calcein uptake

The purpose of this experiment was to compare cellular uptake of calcein from solution and from calcein-loaded liposomes at 37 °C and 4 °C. The experiment was performed as described under Sections 4.3 and 4.4. After Caco-2 cells were allowed to grow for 3 days after seeding, cells were washed and pre-incubated at 37 °C for 30 min with serum-free medium. Then the cells were incubated with either calcein-loaded liposomes or calcein solution (equivalent to 80 μM calcein) at 37 °C and 4 °C for 2 hours. Cells were washed with ice-cold PBS and digested as described under Section 4.3. Measurement of total calcein associated with the cells in each sample was done by spectrofluorometric method at the excitation wavelength of 490 nm and the emission wavelength of 510 nm with appropriate background correction.

4.5.2 Measurement of cell-associated calcein by flow cytometry

The purpose of this experiment was to elucidate the mechanism by which calcein-loaded liposomes were taken up. If the mechanism of uptake was fusion, Caco-2 cells would be high in calcein fluorescence when flow cytometry was used since calcein would be highly diluted in cell cytoplasm. In contrast, if the uptake mechanism was endocytosis, calcein confined in endosomes or lysosomes at high concentration would still be self-quenched. Thus, Caco-2 cells would be low in calcein fluorescence.

The incubation condition of cells with calcein solution or calcein-loaded liposomes was the same as described under Sections 4.3 and 4.4. At the end of incubation period, cells were detached from the culture plate with 0.25% trypsin in 1mM EDTA solution. Trypsin action was stopped with ice-cold PBS. Cells were carefully triturated and transferred to centrifuge tubes. The cell suspension was centrifuged for 4 min at 1,200 rpm in a refrigerated centrifuge (ALC4237R, ALC International, Italy) and the pellet resuspended in PBS. Cells were fixed with an equal volume of 1% formaldehyde.

Total calcein associated with the cells in the sample was measured by a flow cytometer (FACS Calibur, USA) with and argon ion laser light source. The instrument was operated at 488 nm and 15 mW. Calcein fluorescence was collected through a 525/42 nm band pass filter (ISCR, 2004).

4.6 Effect of liposomal composition on calcein uptake by Caco-2 cells

The composition of lipid bilayer and charge on liposomal surface are important parameters influencing the mechanism and extent of liposome-cell interaction. The purpose of this experiment was to compare the extents of calcein uptake from calcein-loaded neutral, positively charged, and negatively charged liposomes. Calcein uptake from solution was used as a control. The experiment was done under the same method and conditions as described under Sections 4.3 and 4.4. Liposome compositions were as described under Section 4.1. The amounts of the three types of liposomes in the incubation medium were comparable since the entrapment efficiencies were rather different.

5. Effect of liposomes on transport of calcein across Caco-2 monolayers

The purpose of this experiment was to investigate whether calcein taken into the cell would undergo exocytosis in appreciable amounts. Neutral liposomes were used since the uptake from this type of liposomes was high. Results from Section 4.6 indicated that inclusion of charged lipid did not increase the extent of calcein uptake into the cells. Amounts of calcein passing through Caco-2 monolayers were compared between calcein solution and calcein-loaded liposomes.

Caco-2 cells were seeded at a concentration of 2.83 x 10^5 cells per 4.71 cm^2 on Transwell® inserts in 6-well culture plates. Caco-2 monolayers were used for transport study when the TEER value exceeded 300 $\Omega \cdot cm^2$. The Caco-2 monolayer on the Transwell® insert was rinsed and pre-incubated with basolateral buffer, pH 7.4, at 37 °C for 30 min on both sides. The pH gradient was not used in this experiment since Caco-2 monolayers were employed here as a representative of any epithelial tissues, not just the

GI tract. The basolateral buffer was used due to its physiologically relevant pH. After pre-incubation, the buffer on the apical side was replaced with the basolateral buffer containing either 100 μM calcein solution or calcein-loaded liposomes at a concentration that gave associated calcein equivalent to 100 μM. Calcein transport was monitored in the apical to basolateral direction during the period between 30-120 min. To confirm the integrity of cell monolayer throughout the experiment, the TEER value was measured again after the experiment was terminated. Measurement of calcein in the basolateral compartment and calcein associated with the cells in each sample was done by spectrofluorometric method at the excitation wavelength of 490 nm and the emission wavelength of 510 nm. Calcein associated with the cells was determined after the cells were washed with ice-cold PBS and digested with 1% Triton X-100. Permeability coefficients and extents of calcein uptake were compared between calcein-loaded liposomes and calcein solution.

6. Effect of liposomes on efflux of rhodamine 123

The purpose of this experiment was to determine the advantage of liposomes in improving uptake and transport of molecules that were substrates of the efflux transporter P-glycoprotein. Rhodamine 123 was used as a P-gp substrate to examine whether liposomes would be able to increase its uptake into Caco-2 cells and to enhance its transport across Caco-2 monolayers.

6.1 Preparation of rhodamine 123-loaded liposomes

Rhodamine 123-loaded liposomes were prepared by the film-hydration method at a total lipid concentration of 50 mg/ml as described under Section 4.1. Briefly, rhodamine 123 (0.1 mg/ml in 4:1 chloroform:methanol) and lipids were dissolved in chloroform: methanol in a round–bottomed flask. The solution was evaporated to a thin lipid film under vacuum for 2.5 hours using a rotary evaporator. The lipid film was hydrated with the aqueous phase (PBS, pH 7.4). The resultant liposomal suspension was examined under a light microscope for quality inspection. The liposome suspension was kept in a

refrigerator at 4 °C until use. Liposome size was reduced to approximately 100 nm with a hand-held extruder as described under Section 4.1. Liposomes were used without further separation of free rhodamine 123 due to the lack of a practical method of separation. The rhodamine 123-loaded liposomes were used within the same day of preparation to avoid liposome leakage and bleaching of the fluorescent marker.

6.2 Effect of liposomes and phospholipid on accumulation of rhodamine 123 in Caco-2 cells

Phospholipids are the major lipids in the plasma membrane of mammalian cells. PC and phosphatidylethanolamine behave as substrates for human P-gp in two multidrug-resistance cell lines transfected with human P-gp (Bosch et al., 1997). Additionally, Lo (2000) reported that these two phospholipids showed some effects on absorption and/or secretion of epirubicin, another P-gp substrate, in Caco-2 cells. The purpose of this experiment was to demonstrate whether liposomes loaded with rhodamine 123 would increase accumulation of the dye in Caco-2 cells. In addition, effect of PC prepared as blank neutral liposomes on the uptake of rhodamine 123 by Caco-2 cells was also studied.

Caco-2 cells were seeded at a concentration of 50,000 cells/ml in 12-well culture plates with a final volume of 1 ml per well. Cells were allowed to grow for 21 days since, from the preliminary experiment, P-gp function was not detected before then. Cells were washed and pre-incubated at 37 °C for 30 min with either serum-free medium or blank neutral liposomes. Then cells were incubated with either (a) rhodamine 123-loaded neutral liposomes, or (b) blank neutral liposomes with rhodamine 123 solution, or (c) rhodamine 123 solution at 37 °C for 2 hours. The concentration of rhodamine 123 was 1.84 μM in all cases. This concentration was selected to give lipid concentration the same as that used in a previous report (Lo, 2000). Cells were washed and digested as described under Section 4.3. Measurement of total rhodamine 123 associated with the cells in each sample was done by spectrofluorometric method at the excitation wavelength of 485 nm and the emission wavelength of 530 nm. Extents of accumulation of rhodamine 123 in Caco-2 cells were compared among these treatments.

6.3 Effect of verapamil on the uptake of rhodamine 123 from liposomes by Caco-2 cells

Verapamil is a specific substrate of P-gp. It has been shown to compete with rhodamine 123 in binding to P-gp. The purpose of this experiment was to test whether liposomes would have any combined effect with verapamil on the uptake of rhodamine 123 by Caco-2 cells. Caco-2 cells were washed and pre-incubated at 37 °C for 30 min with verapamil at 100 μM in serum-free medium. Then cells were incubated with verapamil at 100 μM and either (a) rhodamine 123-loaded neutral liposomes, or (b) blank neutral liposomes with rhodamine 123 solution, or (3) rhodamine 123 solution. The experiment was carried out under the same conditions as that described under Section 6.2. Extents of accumulation of rhodamine 123 in Caco-2 cells were compared among these treatments.

6.4 Measurement of cell-associated rhodamine 123 by flow cytometry

The purpose of this experiment was to confirm the mechanism of uptake of rhodamine 123 from liposomes by Caco-2 cells. Since rhodamine 123 is lipophilic, the dye would not be confined in endosomes or lysosomes after endocytosis. Thus, high rhodamine fluorescence associated with the cells was expected when flow cytometry was used. This would be in contrast with what was expected for calcein.

Caco-2 cells were cultivated for 21 days using the conditions described under Section 6.2. Cells were incubated with either rhodamine 123-loaded neutral liposomes or rhodamine 123 solution at 37 °C for 2 hours. The concentration of rhodamine 123 was equivalent to 1.84 μM for both treatments. After incubation, cells were washed, trypsinized, and fixed before total rhodamine 123 associated with the cells was assayed by flow cytometry as described under Section 4.5.2.

6.5 Effect of liposomal composition on the uptake of rhodamine 123

The purpose of this experiment was to compare the uptake of rhodamine 123 from neutral, positively charged, and negatively charged liposomes. Rhodamine 123 solution was also studied as a control. Compositions of liposomes were as described under Section 4.1. Liposomes were prepared as described under Section 6.1. The experiment was performed as described under Section 6.2 with the three types of liposomes loaded with rhodamine 123 without separation of free dye.

7. Effect of liposomes on transport of rhodamine 123 across Caco-2 monolayers

The purpose of this experiment was to examine whether liposomes would facilitate transport of rhodamine 123 across Caco-2 monolayers. Neutral liposomes were used since they gave highest accumulation of rhodamine 123 in Caco-2 cells. Rhodamine 123 solution was used as a control. The method of experiment was the same as that described under Section 5. The concentration of rhodamine 123 was 18.40 μM. The direction of transport was from apical to basolateral. Transport of rhodamine 123 was monitor during the period of 30-90 min. Permeability coefficients and the extents of uptake of rhodamine 123 were compared between the two treatments.

8. Statistical analysis

Kolmogorov-Smirnov normality test on pooled samples was used to justify the valid assumptions for parametric tests. If the normality test showed that the distribution of data did not significantly deviate from normality, the analysis of variance (ANOVA) with Scheffe or with Dunnett T3 post hoc test was used. Paired T- test was used for comparison of two treatment means.