CHAPTER III

MATERIALS AND METHODS

Materials

1. Ammonium molybdate (Fluka, Switzerland, Lot no. 232685)
2. Calcein (Sigma, USA, Lot no.20K0575)
3. Chloroform, AR grade (Lab scan, Ireland, Lot no. 08041023)
4. Cholesterol (Sigma, USA, Lot no. 58H8567)
5. Dicetylphosphate (Sigma, USA, Lot no. 10K1593)
6. Dimetyl sulphoxide, cell culture grade (Sigma, USA, Lot no. 24K2300)
7. Disposable cell scraper (Greiner bio-one, USA)
8. Dulbecco's modified Eagle's medium (Gibco, USA, Cat no. 12800-017, Lot no. 1227123)
9. Hydrogen peroxide (Merck, Germany, Lot no. K32656587)
10. Fetal bovine serum (Hyclone, USA, Lot no. CSE0443)
11. Fiske-Subbarrow reducer (Fluka, USA, Lot no. 1195556)
12. Griess reagent system (Promega, USA, Lot no. 229601)
13. Lipopolysaccharides (Sigma, USA, Lot no. 016K4133)
14. Methanol (Merck, Germany, Lot no. K36943309)
15. Multiwell plates (Costar®, Corning Incorporated, USA)
16. *N*-Acetyl-L-cysteine (Sigma, USA, Lot no. 046K00951)
17. Penicillin-Streptomycin (Gibco, USA, Lot no. 390947)
18. Phosphatidylcholine (Phospholipon®90 Nattermann Phospholipid GmbH, Germany, Lot no. 770991)
19. Phosphatidylglycerol (Sigma, USA, Lot no. 047K5201)
20. Phosphoric acid (J.T. Baker, USA, Lot no. C07812)
21. Potassium chloride (Merck, Germany, Lot no. TA 419536824)
22. Potassium dihydrogen phosphate (Merck, Germany, Lot no. K23775573707)
23. Polycarbonate membranes (Isopore™, Ireland, Lot no. R7NN07670)
24. Sephadex G-75 (GE Healthcare, Sweden, Lot no. 307356)
25. Sodium chloride (Merck, Germany, Lot no. K33800104441)

26. Sodium bicarbonate (Merck, Germany, Lot no. B870498625)
27. Sodium phosphate, dibasic anhydrous (Merck, Germany, Lot no. F997086532)
28. Sulfuric acid (Analar, England, Lot no. K2361283165112)
29. Tissue culture flasks (Corning®, Corning Incorporated, USA)
30. Triton® X-100 (Sigma, USA)
31. Trypan blue (Sigma, USA)
32. α-Tocopherol (approx. 95%, Sigma, USA, Lot no. 063K0796)

Equipment

1. Analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland)
2. Autoclave (Hiclave™, HVE-50, Hirayama, Japan)
3. Counting chamber (BOECO, Germany)
4. Centrifuge (HETTICH, universal 320R, Germany)
5. Flex column (Kontes, USA)
6. Fluorescence microscope (U-RFLT 50, Olympus, Japan)
7. Freeze dryer (Dura-Dry™MP, Fissystems, USA)
8. Hand-held extruder (LiposoFast™, AVESTIN, Canada)
9. Hot air oven (MEMMERT, Germany)
10. High Performance Liquid Chromatography (HPLC) system equipped with
    - Automatic sample injector (SIL-10A, Shimadzu, Japan)
    - Communicator bus module (CBM-20A, Shimadzu, Japan)
    - Liquid chromatography pump (LC-10AD, Shimadzu, Japan)
    - UV-VIS Detector (SPD-10A, Shimadzu, Japan)
    - Column (BDS Hypersil® C18, 5 µm 300x4.6 mm, England)
    - Precolumn (µBandapack C18, 10µm, 125A°, water Corporation, Ireland)
11. Humidified carbon dioxide incubator (Forma Scientific, USA)
12. Inverted microscope (CKX41, Olympus, Japan)
13. Laminar air flow hood (Microflow, Bioquell, England)
14. Microplate reader (Multiskan MS version 8.0 LABSYSTEMS and Perkin Elmer, wallac 1420, USA)
15. pH meter (Orion model 420A, USA)

16. Rotary evaporator (Buchi, Switzerland)
17. Sonicator (Elma® Transsonic Digitals, Germany)
18. Ultra-filtration devices (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane)
19. UV spectrophotometer (Shimadzu, UV-1601, Japan)
20. Vortex mixer (G-560E, Scientific Industries, USA)

Methods

1. Maintenance of J774A.1 cells

The murine macrophage cell line J774A.1 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, L-glutamine, pyridoxine hydrochloride and sodium pyruvate. The culture medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a CO_2 incubator at 37 °C, 95% R.H. The seeding density was $1x10^5$ cells/$25cm^2$. Subcultures were preformed by scraping when the culture was at 80% confluence.

2. Determination of optimal cell density and lipopolysaccharide concentration for J774A.1 cell stimulation

Nitric oxide (NO), a reactive free-radical gas, can be generated by inducible NO synthase (iNOS) in activated macrophages. One of the most potent activators of macrophages is bacterial lipopolysaccharide (LPS). The purpose of this study was to determine suitable cell density and the lowest concentration of LPS that could activate macrophage cells to produce sufficient amount of NO.

J774A.1 cell suspension (400 µl/well) was seeded in 24-well culture plates at concentrations of $0.5x10^5$, $1x10^5$ and $2x10^5$ cells/ml and cells were allowed to adhere for 48 hours. Thereafter, the medium was replaced with LPS solution in fully supplemented DMEM at various concentrations, ranging from 0.125 to 2.0 µg/ml. Cells were allowed to be in contact with LPS for another 24 hours. The medium was then collected for determination of NO production by Griess reaction (Griess reagent system, Promega®). In principle, NO production is measured in the form of nitrite, a stable reaction product of NO with molecular oxygen. The assay protocol was adopted from that recommended by the manufacturer of the reagent as follows.

An aliquot (100 µl) of cell-free supernatant from each well was transferred to a 96-well microtiter plate and mixed with 20 µl of sulfanilamide reagent. After 10

min, 20 µl of *N*-1-napthylethylenediamine dihydrochloride (NED) reagent was added to the mixture. After another 10 min, the absorbance was monitored at 550 nm using a Multiskan MS version 8.0 (Lab System®) microplate reader. Sodium nitrite was used as a standard to prepare the standard calibration line in the fully supplemented medium. NO concentration was derived from nitrite concentrations back calculated from the absorbance using the standard calibration line.

3. Determination of optimal antioxidant concentrations for NO inhibition in LPS-stimulated J774A.1 cells

The purpose of this study was to examine the optimal concentrations of *α*-tocopherol (TOC) and *N*-acetylcysteine (NAC) that could significantly inhibit NO production without compromising cell viability excessively.

3.1 Preparation of *α*-tocopherol (TOC) dispersion

TOC was dispersed in 0.5% DMSO with 0.025% Tween® 80 using aseptic technique and further diluted to other concentrations with the same medium. The concentration of DMSO was kept at 0.5% to minimize cell toxicity (Napolitano et al., 2005). Sterilization by membrane filtration was not used due to possible adsorption of TOC to the membrane.

3.2 Preparation of *N*-acetylcysteine (NAC) solution

NAC powder was dissolved in Ultrapure® water (Elga®, England) and the pH of solution was adjusted to between 7-8 with 1 M NaOH. The resultant solution was further diluted with Ultrapure® water to the required concentrations. The solution was sterilized by membrane filtration (0.2 µM pore size) when necessary.

3.3 Determination of antioxidant concentrations

J774A.1 cells were seeded in 24-well plates as previously described. After 24 hours, the medium was replaced with either 10% TOC dispersion (final conc. = 0.25–

1.0 mM) or NAC solution (final conc. = 0.1–20 mM). Cells were allowed to be in contact with the antioxidant for 24 hours. The cells were washed with culture medium once and the medium was then replaced with LPS solution (0.125 μg/ml). The supernatant was assayed for NO production in the presence of LPS after 24-hour incubation by Griess reaction as described under Section 2. Afterward, the rest of the medium was removed and MTT solution was added to the cells for cytotoxicity assay as described under Section 3.4. NO production was monitored and compared as percent inhibition as follows.

$$\% \text{ inhibition} = \frac{(\text{NO conc. of Control} - \text{NO conc. of Treatment}) \times 100}{\text{NO conc. of Control}}$$

3.4 Cytotoxicity of antioxidants on J774A.1 macrophage cells by MTT assay

The cytotoxic effect was measured based on the incorporation of the dye MTT by viable cells. The dye MTT (3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) (yellow) is reduced to insoluble MTT-formazan (blue) in mitochondria of viable cells. The assay was carried out as described in a standard textbook on animal cell culture (Freshney, 2000). Briefly, following exposure of the cells to the test compound in the culture medium or to the control culture medium only, the medium was replaced by fresh medium containing MTT (0.4 mg/ml). Then, the culture plate was returned to the incubator for 3 hours to allow the uptake of the dye. After removal of the dye solution, the insoluble MTT-formazan dye and the cells in each well were dissolved with 250 μl of neat DMSO. An aliquot of 25 μl was transferred to a 96-well microtiter plate and further diluted with 75 μl of neat DMSO. The optical density (OD) was read at 570 and 650 nm. The viability was calculated according to the following equation.

$$\% \text{ viability} = (OD_{treatment}/OD_{control}) \times 100$$

4. Preparation of liposomes

4.1 Preparation of TOC-loaded liposomes

The TOC-loaded liposomes were prepared by the film-hydration method at a total lipid concentration of 50 mg/ml for neutral liposomes and 25 mg/ml for negatively charged liposomes. The neutral liposome compositions were phosphatidylcholine (PC):TOC at 96:4 and 92:8 molar ratios. The negatively charged liposomes were composed of PC:dicetylphosphate (DCP) or phosphatidylglycerol (PG):TOC at 86:10:4 and 82:10:8 molar ratios, respectively. Cholesterol (CH) was not added to the preparation to allow maximum incorporation of TOC. The lipids were dissolved in chloroform in a round-bottomed flask. The solution was evaporated to a thin lipid film under vacuum (5 mbar) for 2 hours using a rotary evaporator (Buchi®, Switzerland) The lipid film was hydrated with 5 ml of Ultrapure® water and the resultant liposome dispersion was sonicated using a bath sonicator (Elma® transonic digitals, Germany) at 100% power for 30 min. The liposome dispersion was further extruded through two-stacked 100 nm polycarbonate membranes with a hand-held extruder (LiposoFast™, Avestin, Canada) for 19 cycles. Before being used with cell cultures, the liposome preparations were sterilized by membrane filtration. The entrapment of TOC in liposomes was assayed by spectrophotometric method at 292 nm. The total phospholipid content was assayed by the Bartlett method (New, 1997) as described below (see Section 5.4).

The corresponding blank liposome dispersion was similarly prepared as a control for each TOC-loaded liposome formulation. The blank for the neutral liposomes was composed of 100 mol% PC. Liposomes composed of PC:dicetylphosphate (DCP) or phosphatidylglycerol (PG) at 90:10 molar ratio were used as a control for the negatively charged liposomes.

4.2 Preparation of NAC-loaded liposomes

NAC-loaded liposomes were prepared by the dehydration-rehydration method (New, 1997) at a total lipid concentration of 50 mg/ml for neutral liposomes and 25

mg/ml for negatively charged liposomes. The composition of liposomes comprised PC and CH at a molar ratio of 70:30 for neutral liposomes. For negatively charged liposomes, the compositions were PC, CH and either DCP or PG, at a molar ratio of 60:30:10. TOC (0.1 mol%) was used as an antioxidant in all preparations. From preliminary experiments, TOC at this concentration did not show any significant effect on NO production in LPS-stimulated J774A.1 cells.

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 under vacuum at 40 ^{o}C. The lipid film was kept under vacuum for at least 2 more hours to eradicate traces of the organic solvent. The lipid film was hydrated with 5 ml of Ultrapure® water and then the resultant liposome dispersion was sonicated using a bath sonicator at 100% power for 30 min. The liposome dispersion was lyophilized (Dura-Dry™MP, Fissystems, USA) overnight. When the content was dried, 5 ml of 100 or 200 mM NAC solution was added. The resultant rehydrated dispersion was mixed gently with a vortex mixer until homogeneous. The liposome dispersion was extruded through two-stacked 100 nm polycarbonate membranes with a hand-held extruder. Before being used with cell cultures, the liposome preparations were sterilized by membrane filtration. To determine entrapment efficiency, free NAC was separated from NAC-loaded liposomes by gel filtration using Sephadex® G-75 as a gel matrix. After gel filtration, the amount of NAC encapsulated in liposomes was assayed by high performance liquid chromatography (HPLC) (see Appendix B). The total phospholipids content was assayed by the Bartlett method (New, 1997) as described below (see Section 5.4).

The corresponding blank liposome dispersion was similarly prepared. NAC solution was replaced with Ultrapure® water.

4.3 Preparation of calcein-loaded liposomes

Calcein-loaded liposomes were prepared by the dehydration-rehydration method at a total lipid concentration of 25 mg/ml as described under Section 4.2. The composition of liposomes comprised PC and CH at a molar ratio of 70:30 for neutral liposomes. For negatively charged liposomes, the composition was PC, CH and DCP

at a molar ratio of 60:30:10. The liposome dispersions were prepared following the previously described protocol for NAC-loaded liposomes. The concentration of calcein solution used to hydrate the lipid film was 10 mM. The liposome dispersion was then extruded through two-stacked 100 nm polycarbonate membranes to reduce liposome size to approximately 100 nm. Non-encapsulated calcein was separated from calcein-loaded liposomes by gel filtration using Sephadex G-75. After gel filtration, the resultant liposome dispersion was concentrated back to a total lipid concentration of 25 mg/ml by ultrafiltration devices, (Amicon® molecular weight cut off = 10 K) in a bench-top centrifuge (HETTICH, universal 320R, Germany) at 3000 rpm, 4 °C for 2 hours. The liposome dispersion was sterilized by membrane filtration before incubation with cells. An aliquot of the preparation was assayed for calcein and total lipid contents as described under Sections 5.3 and 5.4.

5. Determination of TOC and NAC encapsulation efficiencies in liposomes

5.1 Quantitative analysis of TOC in liposome preparations

Separation of free TOC from liposome preparation was not necessary since liposome bilayers can accommodate a much higher amount of TOC than the amounts used in this present study (Suntres and Shek, 1994). Five hundred microliters of the liposome dispersion was dissolved in a mixture of methanol:chloroform (4:1) and the solution was diluted to 5 ml in a volumetric flask with the solvent mixture. TOC in the solution was quantified by UV spectrophotometric method at 292 nm (see Appendix B).

5.2 Quantitative analysis of NAC in liposome preparations

An aliquot of liposome dispersion (0.5 ml) was loaded on a pre-equilibrated Sephadex G-75 column (1x25 ml) and eluted with water to separate NAC in liposomes from free NAC. The NAC-loaded liposome fraction was diluted with water to 5 ml. An aliquot of the diluted liposome dispersion was dissolved in 1% solution (final concentration) of Triton® X-100. NAC was quantified by HPLC method

modified from the official method described in the USP29 (see Appendix B). The conditions used were as follows.

Column	:	Thermo Hypersil® BDS C18, 5 µm, 300 x 4.6 mm
Precolumn	:	µBondapack C18, 10 µm, 125A°
Mobile phase	:	50 µM monobasic potassium phosphate adjusted with phosphoric acid to a pH of 3.0
Injection volume	:	20 µl
Flow rate	:	1.5 ml/min
Detector	:	UV detector at 214 nm
Temperature	:	ambient
Run time	:	20 min

The mobile phase was freshly prepared, filtered through 0.45 µm membrane filter and degassed by sonication for 30 min before use.

5.3 Determination of calcein encapsulation efficiency

The calcein-loaded liposome dispersion was diluted with Ultrapure® water and dissolved in 1% Triton®-X 100 (final concentration). The solution was further diluted until the concentration was within the desired range of the standard calibration line. Calcein was quantified using a microplate reader (Perkin Elmer, wallac 1420) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

5.4 Quantitative determination of phospholipids by the Bartlett method (New, 1997)

The phosphorus content of phospholipids was determined by the Bartlett method. In this method, phospholipid phosphorus in liposome dispersion is acid-hydrolyzed to inorganic phosphate and then ammonium molybdate is added to convert the inorganic phosphate into phospho-molybdic acid. The phospho-molybdic acid is reduced to a blue-colored compound by amino-naphthyl-sulphonic acid (Fiske-Subbarrow reducer). The intensity of the blue color is measured

spectrophotometrically, and is compared with calibration standards of phosphate solutions at 800 nm. The phospholipids used in the preparation of liposomes contain one mole of phosphorus per mole of phospholipids.

The liposome dispersion was diluted with Ultrapure® water to give a concentration of approximately 1 mg/ml of phospholipid. Five hundred microliters of the diluted liposome dispersion or the standard solutions was added to separate test tubes, together with a blank (0.5 ml of Ultrapure® water). Each aliquot of the samples and the standard solutions was acidified with 0.4 ml of 5 M sulfuric acid and then incubated at 180-200 °C for an hour in a hot air oven. After the tubes were cooled down, 0.1 ml of 10% v/v hydrogen peroxide was added and the mixture was incubated at 180-200 °C for another 30 minutes. The colorless solutions were cooled down to room temperature. Acid-molybdate solution (4.6 ml) was added to each tube, and the contents were mixed by vortex. An aliquot (0.2 ml) of Fiske-Subbarrow reducer was added to reduce the solutions. The tubes were then covered and placed in a boiling water bath for 7 minutes. After the tubes were cooled down, the absorbances of the blue-colored solutions, including the blank, were measured at 800 nm against water. The molar concentration of phosphorus content was equivalent to the molar concentration of phospholipid content in liposome dispersion.

6. Effects of antioxidant/liposome treatments on NO production of J774A.1 cells

6.1 Effect of blank liposomes on NO production in LPS-stimulated J774A.1 cells

Blank liposomes were prepared by the method described under Section 4.2 (the dehydration-rehydration method). This study was to determine the effects of blank liposomes [neutral liposomes (PC:CH, 70:30) and negatively charged liposomes (PC:CH:DCP or PG, 60:30:10)] at various concentrations of total lipid on NO production by LPS-stimulated macrophage cells. The effect of blank liposomes without CH was also studied at the lipid concentration of 4 mg/ml. The viability of cells treated with blank liposomes was also examined.

J774A.1 cells were seeded in 24-well plates at a density of 1 x 10^5 cells/ml (400 µl/well) and allowed to adhere for 24 hours. Cells were treated with neutral and negatively charged liposomes at 1–4 mg/ml total lipid concentrations or water (as a solvent control) for 24 hours. After incubation, cells were washed with supplemented DMEM without FBS to remove liposomes. Cells were further incubated for 24 hours with LPS solution in fully supplemented DMEM (0.125 µg/ml) to evoke NO production. The amount NO produced in the medium was measured by Griess reaction as described under Section 2. Afterward, the rest of the medium was removed and MTT solution was added for measuring cell viability as described under Section 3.4.

6.2 Effect of antioxidant-loaded liposomes on NO production in LPS-stimulated J774A.1 cells

The experiments were conducted to compare the effect of antioxidant-loaded liposomes with blank liposomes and antioxidant solutions on NO production by LPS-stimulated J774A.1 cells. The viability of the treated cells was also determined to confirm that reduction in NO was not the result of reduction in number of viable cells. Cells were treated with 6 different conditions for each lipid composition as follows:

a) Solvent control
b) Antioxidant solution at low concentration
c) Antioxidant solution at high concentration
d) Blank liposomes
e) Antioxidant-loaded liposomes at low antioxidant concentration
f) Antioxidant-loaded liposomes at high antioxidant concentration

The amount of liposome dispersion used was calculated from the lipid assay to give the final concentration of 4 mg of total lipid/ml in the incubation medium in all cases. NAC concentrations were at 10 and 20 mM since free NAC was not separated out of the preparation. The concentration of TOC in treatments b and c varied according to the encapsulation efficiency of each formulation. The ranges of TOC concentrations were 0.14-0.29 mM and 0.34-0.55 mM for the treatments b and c, respectively.

J774A.1 cells were seeded in 24-well plates at a density of 1 x 10^5 cells/ml (400 µl/well) and allowed to adhere for 24 hours. The treatments and controls were assigned to individual wells and incubated for 24 hours. After incubation, the supernatant in each well was discarded and cells were washed once with DMEM. Cells were further incubated for 24 hours with LPS solution in fully supplemented DMEM (0.125 µg/ml) to evoke NO production. An aliquot of the supernatant was taken for further determination of NO production by Griess reaction as described under Section 2. The rest of the medium was then removed and MTT solution was added to measure cell viability as described under Section 3.4.

6.3 Effect of co-incubation of blank liposomes and antioxidants on NO production in LPS-stimulated J774A.1 cells

The experiments were designed to determine whether blank liposomes could modulate the effect of antioxidant solutions. The viability of treated cells was also determined to confirm that reduction in NO was not the result of reduction in number of viable cells. Cells were treated with 6 different conditions for each lipid composition as follows:

a) Solvent control
b) Antioxidant solution at low concentration
c) Antioxidant solution at high concentration
d) Blank liposomes
e) Blank liposomes with additional antioxidant at low concentration
f) Blank liposomes with additional antioxidant at high concentration

The experiments were performed under the same protocol as described under Section 6.2.

7. Effect of liposome composition on cellular uptake by J774A.1 cells

To determine whether the negatively charged liposomes were taken up into J774A.1 cells better than the neutral liposomes, uptake of calcein-loaded liposomes was used as a surrogate. Calcein is a water-soluble fluorescent dye that undergoes self-quenching at high concentration (>20 mM) and has been used extensively to study liposome-cell interaction (Xiong et al., 2005).

In order to compare cellular uptake between each type of liposomes, calcein was incorporated into liposomes composed of PC:CH (70:30 molar ratio) and those composed of PC:CH:DCP (60:30:10 molar ratio). The dehydration-rehydration method was used to prepared liposomes as described under Section 4.2. Calcein solution for rehydration was used at an unquenching concentration (10 mM). The amount of calcein uptake was monitored quantitatively by fluorospectrometry. The results were interpreted as an indirect measurement of liposome uptake.

J774A.1 cells were seeded at a concentration of $1x10^5$ cells/ml in 24-well culture plates with a final volume of 400 µl/well. Cells were allowed to grow for 24 hours under the normal culture conditions. The medium was replaced with neutral liposomes or negatively charged liposomes at 4 mg/ml total lipid concentration (final concentration) or calcein solution at 0.25 mM (final concentration). The cells were further incubated at 37 °C for 30 min, 2, 4 and 24 hours. At each specified time interval, the medium was removed and cells were washed 5 times with ice-cold phosphate buffered saline (PBS). Cells were digested with 250 µl of 1% Triton®-X 100 at 37 °C for 1 hour. Calcein uptake was measured in each sample using a fluorescence microplate reader (excitation wavelength = 485 nm, emission wavelength = 535 nm). The standard calibration line was prepared in 1% Triton®-X 100 spiked with digested untreated cells. Calcein uptake was expressed as the percentage of calcein detected in the cells calculated from micromole of intracellular calcein per micromole of total calcein.

$$\% \text{ Uptake of calcein} = \frac{\text{Amount of intracellular calcein (µmol)} \times 100}{\text{Amount of total calcein (µmol)}}$$

8. Effect of NAC-loaded negatively charged liposomes on cell viability

Since the severe cytotoxicity was evident only with NAC-loaded negatively charged liposomes, the cause of the cytotoxicity was thus further investigated. The experiment was designed to elucidate whether NAC-loaded negatively charged liposomes without free NAC could exert cytotoxic effect on J774A.1 cells. NAC-loaded DCP liposomes (PC:CH:DCP, 60:30:10 molar ratio) were prepared by the

method described under Section 4.2 and used as a representative preparation for negatively charged liposomes. Non-encapsulated NAC was separated from NAC-loaded DCP liposomes by gel filtration using Sephadex G-75 as gel matrix. After gel filtration, the resultant liposome dispersion was concentrated back to 25 mg/ml total lipid concentration by ultrafiltration method as described under Section 4.3. The liposome dispersion was assayed for NAC and total lipid concentration as described under Sections 5.2 and 5.4, respectively. The liposome dispersion was sterilized by membrane filtration before incubation with cells.

J774A.1 cells were seeded in 24-well plates at a density of 1 x 10^5 cells/ml (400 µl/well) and allowed to adhere for 24 hours. The NAC-loaded DCP liposomes at various concentrations of total lipid (0.5-4 mg/ml) were incubated with the cells for 24 hours. After incubation, cells were washed with supplemented DMEM without FBS to remove the residue of the treatment. Afterward, MTT solution was added to measure cell viability as described under Section 3.4.

9. Effect of negatively charged liposomes on membrane permeability of a model water-soluble compound

The experiments were designed to examine whether negatively charged liposomes and NAC-encapsulated negatively charged liposomes could increase J774A.1 uptake of water-soluble molecules. DCP containing liposomes (PC/CH/DCP liposomes, DCP-liposomes), with and without encapsulated NAC, and calcein were used as representatives of negatively charged liposomes and water soluble molecules, respectively. Blank liposomes were prepared by the dehydration-rehydration method described earlier. NAC-loaded liposomes were prepared using the protocol under Section 4.2. After that, free NAC was separated by gel filtration and the liposome dispersion was concentrated back to 25 mg/ml total lipid using the same protocol as described under Section 8.

Macrophage cells were seeded in 24-well plates at a density of 1 x 10^5 cells/ml (400 µl/well) and allowed to adhere for 24 hours. Cells were treated with blank DCP liposomes or NAC-encapsulated DCP liposomes at 4 mg/ml total lipid concentration along with calcein solution (0.25 mM) or calcein solution at the same

concentration (as a control) for 2 hours. At the end of incubation, the medium was removed from each well and cells were washed 5 times with ice-cold PBS. Cells were digested with 250 µl of 1% Triton®-X 100 at 37 °C for 1 hour. Calcein uptake was measured in each sample using fluorescence microplate reader (excitation wavelength = 485 nm, emission wavelength = 535 nm). The standard calibration line was prepared in 1% Triton®-X 100 spiked with digested untreated cells.

For all experiments involving the cells under Sections 2-9, at least 3 wells of cultured cells were assigned for each treatment condition per each experimental run. Two to three runs, except for the experiment under Section 7 where only one experimental run performed, were performed for each experiment.

10. Statistical analysis

Data analysis was performed on SPSS 13.0. If the test of homogeneity of variances showed that there was no significant deviation of variances in the data, the analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) post hoc test was used. Alternatively, the Dunnett T3 was used if the test of homogeneity of variances indicated significant deviation of the variances. A probability of $P \leq 0.05$ was considered statistically significant.