Chapter IV

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4.1. CHEMICALS

Egg phosphatidyl choline was purchased from Nippon Oils and Fats Co. Ltd, Hyoga, Japan and CSIR Centre for Biotechnology, India. Lymphoprep, BSA, Sepharose-4B, Sephadex G-50, Chloramine-T, Potassium iodide, Sodium metabisulphite, Ethidium bromide, Ammonium molybdate, Ascorbic acid, Dipalmitoyl phosphatidyl choline, Distearoyl phosphatidyl choline, HEPES, (N-2-Hydroxy ethyl piperazine-N’-2-ethanesulfonic acid) and Cholesterol were obtained from Sigma Chemicals Co, USA. The purity of the lipids was checked by thin layer chromatography on silica gel plates. Melphalan was obtained from Wellcome Foundation Ltd, Londra Pomezia (Rome). It was dissolved in phosphate buffered saline (0.2M, pH 7.2, PBS) at a concentration of 3mg/ml just before use. Digitonin, Perchloric acid (AR), Calcein, Hematoxylin, Eosin, Heparin and other chemicals used were of analytical grade purchased from SISCO Research Laboratories, Bombay, India. Streptomycin and Benzoyl penicillin were purchased from HiMedia Pvt. Ltd., Bombay. The organic solvents used were HPLC grade purchased from Qualigens, India. Dulbecco’s Modified Eagle’s media, Fetal Calf Serum (FCS) and RPMI 1640 were purchased from Gibco, USA. Na\(^{125}\)I specific activity 5.2 Ci/mimole was purchased from Board of Radiation and Isotope Technology (BRIT), Bombay.

4.2. ANIMALS AND TUMORS

C57Bl/6 mice were purchased from National Institute of Immunology, New Delhi, India, or from National Institute of Nutrition, Hyderabad. B16F10 murine
melanoma was a gift from Dr. Vincent Hearing; National Institutes of Health, MD, USA. 3\times 10^6 melanocytes in 50\mu l PBS were injected subcutaneously (s.c) in the shank region of 10 to 12 weeks old male C57Bl/6 mice. The tumors were allowed to develop for 15 to 20 days.

Tumor size was determined by measuring the tumor volume using the formula \( V = \frac{\pi}{6} \times D_1 \times D_2 \times D_3 \) where "D1,D2,D3" represent three orthogonal diameters.

4.3. METHODS

4.3.1. PREPARATION OF SMALL UNILAMELLAR VESICLES

Small unilamellar vesicles (SUVs) were prepared by the method of Kirby and Gregoriadis (1984). Briefly egg phosphatidyl choline and cholesterol (7:1 molar ratio), 25mg of total lipid /ml of organic solvent or DPPC:DSPC (9:1 molar ratio) were dissolved in chloroform : methanol (2:1 v/v), evaporated under reduced pressure and dried by over night dessication. The lipid film was suspended in 200\mu l of distilled water and sonicated using a probe type sonicator (Heat systems Ultrasonics W-385, USA) at 100mA, 1 pulse/sec for 30 min with intermittent cooling. Melphalan was suspended in PBS (3mg/ml), flash frozen and lyophilised. 0.2ml of distilled water was added and vortexed for 20 min. The volume was made up to 2ml and centrifuged at 105,000 g for 1hr at 4\degree C. Liposomal pellet was suspended in 0.5M NaCl. For preparation of calcein entrapped liposomes the lipid film prepared as mentioned above was suspended in 2ml of 100mM calcein in PBS and sonicated as above. The unentrapped calcein was removed by passing the liposomal suspension through Sepharose-4B column. The column
(80 x 0.5cm, 50ml bed volume) was pre-equilibrated with phosphate buffered saline pH 7.2, (0.2M). Uniform flow rate of 12ml/hr was maintained using a peristaltic pump (Pharmacia, LKB Pump P1). 1 ml fractions were collected using a fraction collector (Pharmacia FRAC 100). The fluorescence intensity of the fractions was recorded using a spectrofluorimeter (Jasco FP 777, Japan) at an excitation \( \lambda_{\text{max}} \) of 545nm and emission \( \lambda_{\text{max}} \) of 566nm. Ethanol 6%(v/v) was added to the liposomal preparations just before use and their thermotropic behaviour was studied by Differential Scanning Calorimetry (DSC). Alternatively liposomes were prepared using Liposomat (DIANORAM, Germany).

Liposomat usage helped in the preparation of homogeneous, stable, small unilamellar vesicles of defined, uniform size.

4.3.2. DETERMINATION OF Tm BY DIFFERENTIAL SCANNING CALORIMETRY

Thermotropic behaviour of liposomal preparation was studied by differential scanning calorimetry (DSC-10, Du-Pont, TA Instruments 2000, USA) equipped with a thermal controller. The phase transition temperature (Tm) of 10\( \mu \)l of liposomal suspension containing 0.15\( \mu \)moles of phospholipid and 6\( \mu \)l of ethanol was determined at a heating ramp rate of 5\(^\circ\) C/ min. The phosphorus content of the liposomal preparations was determined by McClare’s method (1979).
Insulated container

Electronics to regulate heat to sample and reference

Heaters

Temperature sensors

Lipid vesicle suspension (sample)

Buffer blank (reference)

Insulated container

Data recorder

Differential scanning calorimeter
4.3.3. THIN LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS.

The purity of egg phosphatidyl choline used for the preparation of liposomes was checked by TLC on silica gel plates. 0.5 mg of phospholipid was dissolved in 1ml of (2:1v/v) chloroform: methanol. 10ul of lipid solution was applied to the TLC plate as a single spot (2cm from the bottom, the plate was dried in a stream of air thereafter placed in a chromatographic chamber). The lipids were resolved using a solvent system comprising of chloroform: methanol: water: ammonia (70:30:3:2 v/v). Thereafter, the plates were dried in a stream of air and placed in the iodine chamber for the detection of lipid spots. Lipids appeared as yellowish spots on a yellowish-white background.

4.3.4. DETERMINATION OF PHOSPHOLIPID CONTENT OF LIPOSOMES

200ul of liposomal preparation was treated with 1N HCl (pH 1.5) and the volume was made upto 1ml with distilled water. Lipids were then extracted by vortexing with 1ml of chloroform: methanol (2:1v/v). 10ul of organic phase (lipid extract) was applied to the TLC plate as a single spot. The plate was eluted in the solvent system comprising of chloroform: methanol: water: ammonia (70:30:3:2 v/v). Lipid spots were detected by exposure to iodine and scrapped. Total phosphorus was determined by scrapping off silica gel from the plate and extracting with the following solvent systems sequentially: (1) 3ml of chloroform: methanol: water (65:24:4 v/v), (2) 2ml of chloroform: methanol: water (65:25:4 v/v), (3) 2ml of methanol, (4) 2ml of methanol: acetic acid: water (94:1:5 v/v). After extraction, the silica gel was separated by centrifugation and the pooled extracts were transferred to a 10 ml measuring flask and volume was made
up by the addition of chloroform: methanol: water (65:25:4 v/v). Aliquots of this solution were transferred to reagent tubes, and phosphorus was assayed by modified McClare's method (1979).

4.3.5. ESTIMATION OF PHOSPHORUS BY McCLARE'S METHOD

An aliquot of the sample was pipetted into a 15 ml glass stoppered tube and 4 ml of triple distilled water was added to it. The sample was digested by evaporating to complete dryness in a thermostated incubator set at 210±5°C for 30 min. 0.4 ml of perchloric acid (60%) and 0.2 ml of 0.2% ascorbic acid were added to the tubes and refluxed by heating at 60°C for 30 min in a water bath. The blue color developed was read at 600 nm in a spectrophotometer after cooling the tubes.

4.3.6. SIZE DETERMINATION BY SCANNING ELECTRON MICROSCOPY

4.3.6.1. Preparation of liposomal suspension for scanning electron microscopy

Two drops of liposomal suspension were fixed in 0.1M phosphate or sodium cacodylate buffer containing 2% glutaraldehyde for 10 min. It was centrifuged at 1000 g for 5 min and washed within distilled water. The supernatant was discarded and the sample was repelleted using distilled water. The pellet was resuspended in distilled water and a thin film was applied on cover slip followed by air drying. The sample was sputter coated with gold.
* Metal film coating of suspension

The metal coating of the SEM specimen namely liposomal suspensions was carried out under vacuum in an inert atmosphere using argon gas. A coating of uniform thickness was obtained by evaporating the metal (target) onto the liposomal suspension kept at specified distance (cathode specimen distance). Using gold as a target a coating of about 35nm (350Å) thickness can be obtained under the following conditions (Balzers SCD 020 Sputter device):

**Current**: 21.5mA

**Pressure**: 0.05m bar

**Gas (atmosphere)**: Argon

**Distance cathode specimen**: 30 mm

**Time**: 1 min

Size of small unilamellar vesicles was determined using scanning electron microscope (Philips Netherland, 501-B).

4.3.7. TRANSMISSION ELECTRON MICROSCOPY

4.3.7.1. Hyperthermia mediated in vitro drug release from thermosensitive liposomes

The liposomal suspensions entrapping melphalan were heated at 43°C for 1 hr. Thereafter, these suspensions were applied to formvar coated grids (200 mesh) and allowed to settle for 30 sec. Samples were stained with 1% phosphotungstic acid (PTA) and after 30 sec the solution was dried. A transmission electron microscope was used
to observe the release of melphalan from the liposomal suspensions.

4.3.8. EFFECT OF SERUM ON STABILITY OF THERMOSENSITIVE LIPOSOMES

200μl of thermosensitive liposomes entrapped calcein and 25% of human serum or 10mM HEPES buffer (pH 7.4) containing 0.9% NaCl, were mixed and incubated at 37 or 43 °C for 1h. Thereafter, 200μl of the mixture was withdrawn and applied to a Sephadex G-50 column (0.65x10 cm). The column was eluted with 10mM HEPES (pH 7.4)/0.9% NaCl, and 1ml fractions were collected. The fluorescence intensity of the mixture was measured with a fluorescence spectrophotometer Jasco FP-777, JAPAN, at an excitation maxima λmax. of 545 nm and emission maxima λmax. of 566 nm.

4.3.9. LIPID TOXICITY STUDIES

The lipid toxicity of the liposomal preparations was evaluated using normal human peripheral blood mononuclear cells. 0.25ml of heparinized blood was diluted with an equal volume of RPMI 1640 (90%), supplemented with fetal calf serum (FCS) 10% and antibiotics, streptomycin (100μg/ml) and benzoyl penicillin 100 units/ml. The diluted blood was layered on an equal volume of lymphoprep and centrifuged at 1000 g in Sorvall RC-3B for 30 min. The buffy coat was separated and recentrifuged. The cell pellet was suspended in 1 ml RPMI 1640 and counted. 1.5x10⁶ cells/1.5ml medium were plated in 60mm petridishes and incubated at 37°C. Liposomes (100-2500 n mol phospholipid) were added and cells were incubated at 37°C for 1h. After liposome treatment, medium was
removed, cells were washed and $^3$[H]-Thymidine (1.5$\mu$Ci)/1.5ml RPMI 1640 medium was added to each petridish. Cultures were incubated at 37°C for 24h. Thereafter, cells were scrapped, filtered, washed and counted using Bray’s scintillation fluid. Control untreated cultures were incubated at 37°C throughout the experiment.

4.3.10. IN VITRO CYTOTOXICITY STUDIES

Melanocytes were grown as monolayer cultures in 60mm petri dishes in MEM 90%, supplemented with fetal calf serum 10% and antibiotics streptomycin and benzoyl penicillin in a humidified atmosphere of 5% carbon dioxide, 95% air at 37°C. Cells in log phase of growth were harvested, suspended in PBS and counted using haemocytometer under light microscope. Cells (3X10⁶/ml PBS) were given the following treatments

(i) untreated control melanocytes were incubated at 37°C throughout the duration of the experiment, C;
(ii) 200μl suspension of empty liposomes (3.75μ moles phospholipid), EL;
(iii) hyperthermia 43°C, 1hr, HT;
(iv) melphalan (3μg/ml), CT;
(v) radiation 1.5 Gy, RT;
(vi) melphalan entrapped thermosensitive liposomes in combination with hyperthermia 43°C, 1hr, HT+L.

Thereafter the cells were pelleted and cell death was measured by spectrofluorimetric cell death assay of Beletsky and Umansky (1990). This method is
based on binding of ethidium bromide to DNA of dead cells and subsequently permeabilizing the cells and staining the whole cell population. It measures cytotoxic and cytostatic effects simultaneously. Ethidium bromide (10μg/ml) was added to equal number of cells in PBS and incubated for one hour at 37°C. The excitation maxima was observed at 306nm and emission maxima was observed at 618nm. Thereafter digitonin was added to the cell suspension (10μg/ml) and incubated at 37°C for 1h. The fluorescence measurement was repeated and total cell fluorescence was obtained. Percentage killing was calculated using following equation: Fv = Ft - Fd where Fv is fluorescence of viable cells, Ft, Fd represent fluorescence of total and dead cells respectively.

\[
\text{% cell viability} = \frac{Fv}{Ft} \times 100
\]

\[
\text{% cell killing} = 100 - \text{% cell viability}
\]

4.3.11. HYPERTHERMIA TREATMENT

HT was given by immersing the tumor bearing leg of animals in a circulating water bath maintained at 43±0.2°C using a specially designed plexi glass holder wherein only the tumor bearing leg was in contact with water. The mice were air cooled during the heat treatment. The temperature in the centre of the tumor was measured with a small thermistor probe (0.8mm diameter) [Tastomed P, Braun Electronics, Frankfurt, West Germany]. The tumor temperature rose within 5 min from 34 to 43°C. Rectal temperature increased from 37 to 39°C and varied by 0.5°C during the heating.
4.3.12. TEMPERATURE DEPENDENT RELEASE OF ENTRAPPED CALCEIN FROM THERMOSENSITIVE LIPOSOMES IN VIVO

Melanoma bearing mice were given i.v. injections of 200μl of calcein entrapped liposomes followed by immediate heating of the tumor bearing leg at 43°C for 1 hr. Corresponding control experiments were conducted by injecting calcein entrapped vesicles in unheated group of animals maintained at room temperature. After one hour the animals were perfused with heparinised buffer and were sacrificed. Tumors were excised and processed for histopathological studies. The tumor tissue was fixed in 10% neutral buffered formalin, processed into paraffin blocks, sectioned at 5μm, and stained with hematoxylin and eosin for histopathological examination. The uptake of liposomal calcein by tumors and its release from liposomes by heat was observed using a fluorescence microscope (Nikon OPTIPHOT-2-EF-D).

For Hematoxylin and eosin staining, 5 micron cryosections were fixed in acetone for 2 min, transferred to alcohol for 1 min and washed under slow running water for 2-3 min. Washed sections were dipped in hematoxylin for 1 min for staining the nucleus. Excess stain was washed off by dipping the slides in 1% acid-alcohol and then keeping it under slow running water for 2-4 min. The slides were examined under light microscope for any excess staining. If there was nonuniform staining in any section, it was dipped again in 1% acid-alcohol and washed properly under slow running water. Slides were then sequentially dipped in eosin (30, 50, 70, 90%) to stain the cytoplasm. The slides were transferred to acetone and finally washed with varying grades of alcohol. After 95% alcohol washing, the slides were dipped in xylene for 2 min,
dried, glycerine mounted, viewed under a light and fluorescent microscope and photographed.

4.3.13. VASCULAR PERFUSION

Vascular perfusion was carried out intra cardiacally using a transfusion set (i.v. set) at room temperature. The animal was anaesthetised with chloroform and the heart was exposed. The ascending aorta was canulated to about 1mm internal diameter through an incision in the left ventricle. Care should be taken not to let in air while conducting perfusion. Initially, 0.5ml (2500 U) heparin was allowed to flow rapidly through the tail vein to prevent blood clotting during perfusion for 5min. The right auricle was incised for the out flow of the blood. The rate of flow was then induced to about 6 to 8ml/minute. As the blood is displaced from all the organs these progressively appear paler in colour.

4.3.14. TUMOR LOCALIZATION OF M-ThLip BY TRANSMISSION ELECTRON MICROSCOPY

Melanoma bearing mice were given (i.v.) injection of 200µl of M-ThLip followed by immediate heating of the tumor bearing leg at 43°C, 1hr, while tumor bearing animals in control group receiving M-ThLip were kept at 37°C for the same time period. Thereafter the animals were perfused as described above (4.3.13) and sacrificed. Tumors were excised and processed for block preparation for Transmission Electron Microscopy (TEM). The tissues were processed for TEM by the method of Nalin et
al. (1989). The animal was sacrificed either by anaesthetization or decapitation, dissected and tumor tissue was taken. The tissue was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 6.8) for 30 min at 4°C. After fixation it was rinsed thrice in phosphate buffer. Post fixation the tissue was dipped in 1% osmic acid in phosphate buffer for 1 hr at 4°C and was rinsed in phosphate buffer 2-3 times at room temperature. Dehydration of tissues in 50, 70, 95% acetone was carried out sequentially for 30 min each at 4°C. Infiltration was done by taking one part of absolute acetone and one part of embedding medium for 2 h at room temperature with the lid of the tube slightly open.

* Embedding medium

1. DDSA (Hardner) : 15ml
2. Epon 812 : 5.5ml
   (Highly viscous resin)
3. Araldite : 5.0 ml CY 212
   (Epoxy resin)
4. DMP : 1ml (accelerator)

DDSA, Epon 812 and Araldite were mixed well and kept at room temperature overnight. CY212 was added before embedding and mixed thoroughly.

The tissue was kept overnight with the lid of the container slightly open. The tissue was embedded in a beam capsule (or any other mould) and blotted on a filter paper to eliminate traces of acetone. The blocks were labeled, polymerized at 60°C for 24 hrs and
ultra thin sections were cut using Reicherts microtomes). These sections were stained with toludine blue.

For staining of the grid the sections were picked on the matted side of the grid and dried using a filter paper from beneath. Few drops of uranyl acetate (saturated solution in 50% alcohol) were added on to the parafilm kept at different places in a petridish. Grids were placed over the same with the section facing down (the grid should float on the surface of the drop) for 10 min at room temperature. The grids were washed in distilled water using a syringe and jet of water spray. Drying was carried by holding the grids in a slanting position. 50 mg lead citrate was added to 12ml of distilled water containing few drops of NaOH and placed over the parafilm in a petridish. The grids were immediately transferred to the lead citrate drop on parafilm and stained for 10 min at room temperature. The grids were washed in distilled water, dried on a filter paper and viewed under a transmission electron microscope (Philips Netherland, 501-B).

4.3.15. TISSUE DISTRIBUTION OF Na\(^{125}\)[I] BSA ENTRAPPED THERMOSENSITIVE LIPOSOMES IN MELANOMA BEARING MICE

Iodination of BSA was carried out by chloramine-T method (Hunter and Greenwood, 1962). Briefly, 100\(\mu\)l of BSA (1mg/ml) was incubated with 1mCi-\(^{125}\)[I] and 10\(\mu\)l of chloramine T (1mg/ml) at room temperature for 2-4 min. Thereafter 10\(\mu\)l of sodium metabisulphite (2mg/ml) was added and incubated for 2 min. 10\(\mu\)l of potassium iodide (5x10\(^{-3}\) M) was added and free iodine was removed by passing through Sephadex
G-50 column equilibrated with 0.25% BSA. Phosphate buffered saline (0.2M), pH 7.2 was used as eluting buffer. 0.5ml fractions were collected using a fraction collector and a flow rate of 5ml/hr was maintained using a peristaltic pump. 50μl aliquots were taken for TCA precipitation (5% final concentration) and 50μl aliquots were counted in gamma counter (Isodata 100 Series, Okidata).

Na$^{125}$I-BSA was entrapped in ThLip as described above for the entrappment of melphalan. Melanoma bearing mice (6 mice/group) were given i.v. injection of Na$^{125}$I-BSA entrapped thermosensitive liposomes ($1.22 \times 10^6$ cpm/200μl). The animals were kept for 1hr and 4hrs respectively at room temperature. Both the groups of animals were distributed into two batches, one batch of each group was given hyperthermic treatment at 43°C for 1hr. Thereafter, animals were perfused using ice cold saline and major organs like heart, liver, kidney, spleen, lungs, and tumor were excised and weighed. The organs were taken for measurement of radioactivity using a gamma counter.

4.3.15.1. Blood clearance of Na$^{125}$I-BSA liposomes.

The blood clearance rate of liposomes entrapped $^{125}$I-BSA was measured after i.v. administration of temperature sensitive liposome entrapped Na$^{125}$I-BSA to tumor bearing mice with or without HT (43°C). Retro-orbital bleeding was carried out serially at 1hr and 4hrs respectively. The radioactivity in blood samples withdrawn at different time intervals was measured using gamma counter.
IN VIVO RADIOTHERMOCHEMOTHERAPY STUDIES USING NATURAL LIPID DERIVED LIPOSOMES

Treatment regimes

Experiments were performed on day 14-15 after inoculation of murine melanocytes in C57Bl/6 mice as described earlier section (2.2). On day 15 animals were randomized into following groups (6 mice/group).

(a) Untreated controls, C;
(b) Hyperthermia 43°C, 1 hr, HT;
(c) Melphalan in solution (3mg/Kg body weight), CT;
(d) A combination of melphalan in solution (3mg/kg body weight), CT and hyperthermia 43°C, 1 hr, CT+HT;
(e) Thermosensitive liposomes (prepared from natural phospholipid) encapsulated melphalan (M-ThLip) without HT, (M-ThLip - HT);
(f) Thermosensitive liposomes encapsulated melphalan and hyperthermia 43°C, 1 hr, (M-ThLip+HT);
(g) Radiation (900cGy) RT;
(h) Radiation (900cGy) with melphalan (3 mg/kg body wt), RT+CT;
(i) Radiation with HT 43°C, 1 hr , RT+HT;
(j) Radiation in combination with M-ThLip and HT (M-ThLip+RT+HT).

The amount of melphalan entrapped in ThLip was equivalent to that used in the group receiving free drug in solution. Initial tumor dimensions were recorded before...
commencement of the treatment.

For radiothermochemotherapy studies, 250μl of liposomal suspension (3.75 μmoles phospholipid) containing 60 μg melphalan was injected into the tail vein of the mice. The mice which received HT or injection of M-ThLip+HT as mentioned above were transferred to plexi glass holders for HT treatment. HT was given within 30 sec of injection of M-ThLip. In the groups receiving HT in combination with RT, HT was given after RT treatment in order to kill the radioresistant population of cells. Heating was continued for 1hr. Thereafter tumor volume was measured. The treatments were repeated on alternate days for a period of two weeks. Tumor volume was measured daily for a period of one month. The survival of the animals was monitored for three months. Each experiment was repeated three times.

4.3.17. PREPARATION AND DETERMINATION OF PHASE TRANSITION TEMPERATURE OF SYNTHETIC LIPID DERIVED LIPOSOMES

Small unilamellar vesicles were prepared using 25 mg of dipalmitoyl phosphatidyl choline and distearoyl phosphatidyl choline (9:1 molar ratio)/ml of chloroform:methanol (2:1 v/v) as explained earlier in section (4.3.1). The thermotropic behavior of these small unilamellar vesicles was studied by differential scanning calorimetry (DSC). 10μl of liposomal suspension containing 0.15 μmoles of phospholipid was heated at a ramp rate of 5°C/min in DSC to study the melting temperature of the lipids used for liposomal preparation.
4.3.18. **IN VIVO RADIOTHERMOCHEMOTHERAPY STUDIES USING SYNTHETIC LIPID DERIVED LIPOSOMES**

The in vivo efficacy of ThLip entrapped melphalan (prepared from synthetic lipids viz., DPPC and DSPC) on murine melanomas was determined using similar treatment regimes as described in section (4.3.16) for evaluation of in vivo efficacy of drug entrapped in natural lipid derived ThLip.

4.3.19. **STATISTICAL ANALYSES**

Differences in medians among treatment regimes within each group were tested using Kruskal Wallis test (Conover, 1950). Multiple comparisons were performed when significant differences were found. Fruends test was used to find the significant changes within each group at different days after tumor implantation.