Electronic Supplementary Material (ESI) for Integrative Biology

Supplemental information

Materials and methods

Encapsulation efficiency of paclitaxel

In order to determine the encapsulation efficiency of paclitaxel in nanovesicles, 100µl aliquot of LN-PTX was mixed with 900µl of methanol in order to break open the nanovesicles. The solution was then passed through a 0.22 µm filter and analysed by reverse-phase HPLC method (Agilent 1100 Binary LC pump liquid chromatograph, Zorbax SB C-18 column, 250×4.6mm, 5µm). The mobile phase was acetonitrile-water (60:40, v/v), injection volume was 20µl and the column temperature maintained at 25ºC. The analysis was performed at a flow rate of 1.5 ml/min with the UV detector at 227 nm. Encapsulation efficiency (EE %) was calculated using below formula:

Encapsulation efficiency (EE %) = $(W_t/W_i) \times 100$ %

where W_t is the total amount of drug in the nanovesicle suspension and Wi is the total quantity of drug added initially during preparation.

In vitro release of paclitaxel from LN-PTX

In vitro release study of paclitaxel was performed by dialysis bag method at pH 7.4 and 37^{o}C temperature conditions. Dialysis was done by using HIMEDIA® LA 387 Dialysis Membrane-50 (molecular wt. cutoff 5000-10000) against 25 % methanolic solution of PBS as release medium in USP dissolution apparatus type-II (Electrolab, TDT-08L). Choice of the release medium was based on adequate solubility of paclitaxel while maintaining as much PBS as possible. The release study was done with a sufficiently good sink condition. Briefly, sealed dialysis bag (6 cm) containing the formulation (paclitaxel amount 2.73 mg) was placed in 150 mL of the release medium at specified pH and temperature conditions. The release medium was stirred at 100 rpm to enhance the solubility of paclitaxel and prevent the formation of a stagnant layer at the membrane and outer solution interface. 2 mL aliquots were taken from the medium at predetermined intervals and replaced with an equal volume of fresh medium. This replenishing assured the maintenance of good sink conditions. During the entire release process, no precipitation or flocculation was observed by visual inspection in the release medium. Paclitaxel present in the withdrawn aliquots was quantified by UV-spectrophotometry (Perkin Elmer Lambda 25) at 228 nm after appropriate dilutions.

Surface activity measurements

Langmuir–Blodgett instrument (KSV Instrument Ltd., Finland) was used for surface activity measurements of LN-PTX, LN-B and Taxol® in order to understand their adsorption patterns at the pulmonary air-aqueous interface. A small trough (2 cm in diameter) of Teflon was used for adsorption studies. 3ml of the formulation was added to this trough. The trough was cleaned with methanol after each experiment. Trough temperature was maintained at 37ºC by an external circulating water bath (Pooja Lab Equipments, India). Surface pressure was measured continuously by a gold Wilhelmy plate connected to a microbalance for surface pressure measurement. The adsorption of the nanovesicles into the air–liquid interface leads to changes in surface pressure with respect to time. The adsorption was studied for 30 minutes for each sample. All the experiments were done in triplicates to ensure reproducibility.

Airway patency

Capillary surfactometer (CS) from Calmia Biomedicals (Toronto, Ontario) was used to study the airway patency and surfactant ability of LN-PTX. Briefly, 0.5 µl of the sample to be evaluated is introduced into the narrow section of the glass capillary, where the I.D is 2.5 mm, similar to the terminal airway. Capillary, at its one end is connected to bellows and a pressure transducer. Pressure is raised and recorded when bellows are slowly compressed. The increasing pressure results in the passage of the sample from the narrow section of the capillary, allowing air to get through it which results in the abrupt lowering of the pressure. If the sample contains well functioning pulmonary surfactant the sample liquid will not return to the narrow section and the capillary remains unoccluded. The airflow generated by the compressing bellows will not meet any resistance in the capillary and hence the pressure recorded will be zero. If on the other hand, the sample does not contain well functioning pulmonary surfactant the sample liquid will return repeatedly resulting in significant resistance to the airflow and hence no lowering of the pressure.

***In vitro* lung deposition**

In vitro lung deposition studies for LN-PTX and Taxol were performed using glass twin impinger apparatus (Copley Scientific, Nottingham, UK), adapted from apparatus A of European and British Pharmacopoeia. Formulations (1 mg PTX/ml, 7 ml) suspension was placed in the sample holder and aerosolized using Omron Micron AIR U22 Ultrasonic Nebulizer. 60 L/min air flow rate was maintained inside the impinger during nebulization by a vacuum pump. In the glass twin impinger, 7 and 30

ml of acidified methanol (200 µl of glacial acetic acid to 1 L methanol) was taken in upper and lower chambers respectively. Nebulization was done for 1 minute and at the end, samples from throat, upper stage (stage I) and lower stage (stage II) were collected and quantified for paclitaxel using HPLC (Agilent 1100 Binary LC pump liquid chromatograph, Zorbax SB C-18 column, 250X4.6 mm, 5 µm). The mobile phase was acetonitrile-water (60:40 v/v) and the column temperature was maintained at 25^{o}C. The analysis was performed at a flow rate of 1.5 ml/min with UV detector at 227 nm.

***In vitro* cytotoxicity**

At 70-80 % confluency, cells were harvested and seeded onto 96 well tissue culture plates (NUNC, USA) at a density of 10^4 cells per well as 200 µl suspension. Plates were incubated for 24 h in saturated humid conditions at 5 % CO_2 and 37^{o}C. Spent medium from each well was then removed and plates were incubated with graded concentrations of the formulations at 37^{o}C in 5% CO_2 incubator for 72 h. Cells treated with medium only, served as control. At the end, SRB (Sulphorhodamine B) (Sigma Aldrich, Mumbai , India) assay was conducted. In brief, old media with the formulation was discarded and 200 µl complete media was added. Then cells were fixed by adding 50 µl of ice-cold 50% trichloroacetic acid (TCA, Loba Chemie, India) slowly to the medium and incubated at 4 °C for 1 h. Further, plates were washed five times with deionized water and dried in air. 100 µl of 0.4% sulphorhodamine B dissolved in 1% acetic acid was added to the fixed cells and kept at room temperature for 20 minutes after which they were washed with 1% acetic acid to remove unbound dye. The plates were dried and 100 µl of 10 mM Tris base was added to each well and kept for 20 min to solubilize the dye. Thereafter, the plates were placed on a shaker to allow mixing and the absorbance (OD) of each well was read in a plate reader (Thermo Electron Corporation, USA) at 560 nm. Cell viability was measured using the formula:

% viability = Absorbance of sample/Absorbance of control x 100.

Cellular uptake

70-80 % confluent cells were seeded onto glass coverslips placed in 24 well tissue culture plate (NUNC, USA) at a density of 10^5 cells per well as 1 ml volume of the suspension. Plates were incubated at 37^{o}C in 5% CO_2 incubator for 24 h. Spent medium from the wells was then replaced by fresh medium containing either

rhodamine-6G or rhodamine-6G loaded nanovesicles at final rhodamine-6G concentration of 1.6 µM. Following an incubation of 1 hour, medium was removed from the wells and washing was done 10 times with phosphate buffered saline (PBS). 1 ml of 10% formaldehyde solution in PBS was then added to each well and was retained there for 10 minutes in order to fix the cells that were adhered on the surface of the coverslips. Coverslips were then placed on the glass slide over glycerol mount in order to prevent the cells from drying. Slides were observed by a confocal laser scanning microscope (CLSM) (Olympus Fluoview, FV500, Tokyo, Japan) using an excitation wavelength of 570 nm and emission wavelength of 590 nm for rhodamine 6G and images were acquired and analyzed with 60X water immersion objective using the Fluoview software (Olympus, Tokyo, Japan).

Mechanism of cellular uptake and interaction

Confluent cells at a density of 10^5 cells per well were seeded in 24 well tissue culture plate (NUNC, USA) and incubated for 24 h at 37^{o}C in 5% CO_2. Subsequently, spent medium in some wells was replaced by fresh medium containing 0.1 % sodium azide and the plate was further incubated for 30 minutes. One separate plate was incubated at 4^{o}C for 30 minutes. Following this, spent medium in all the plates and wells was replaced by fresh medium containing rhodamine-6G loaded nanovesicles at final rhodamine-6G concentration of 1.6 µM and the plates were further incubated at 37^{o}C (with or without 0.1 % azide) or 4^{o}C. After 0.5 h, 1 h and 3 h time points, spent medium from the wells was removed and washing was done 10 times with PBS. Cells were lysed by adding lysis buffer (1 % Triton X-100 and 2 mM EDTA) and plates were read by a fluorescence plate reader (Victor 3V Multilabel Plate Reader, PerkinElmer, USA) for rhodamine-6G content (λ_{ex} = 485 nm and λ_{em} = 535 nm). Rhodamine-6G content in each well wad normalized by cellular protein content as determined by Pierce BCA protein assay kit (Thermo Scientific, Pierce, USA).

Flow cytometry

Confluent cells at a density of 5 × 10^5 cells per well were seeded in 24 well tissue culture plate (NUNC, USA) and incubated for 24 h at 37^{o}C in 5% CO_2. Subsequently, spent medium in some wells was replaced by fresh medium containing 0.1 % sodium azide and the plate was further incubated for 30 minutes. One separate plate was incubated at 4^{o}C for 30 minutes. Following this, spent medium in all the plates and wells was replaced by fresh medium containing rhodamine-6G loaded nanovesicles at final rhodamine-6G concentration of 1.6 µM and the plates were further incubated at

37°C (with or without 0.1 % azide) or 4°C. After 1 h, spent medium from the wells was removed and washing was done with PBS followed by trypsinization of cells. Cells were then centrifuged, resuspended in PBS and analyzed using BD FACSAria (SOS) flow cytometer (BD Biosciences, USA) by taking 10000 events. Data was analyzed by FCS Express software. Cells treated with free Rh-6G at 37°C were also evaluated in the same way.

Apoptosis assay

Briefly, confluent A549 cells at a density of 10^6 cells per well were seeded in 6 well plate (NUNC, USA) and incubated for 24 h at 37°C in 5% CO_2. After attachment, medium was removed and cells were further incubated for 48 h in the presence of LN-PTX, LN-B and Taxol® separately at equivalent paclitaxel concentration of 1 µM. Cells incubated with medium alone were used as control. Further, spent medium and trypsinized cells were combined and centrifuged to obtain the cell pellet. Cells in the pellet were lysed for 3 h at 60°C using lysis buffer (50 Mm Tris HCl, pH 8.0, 10 mM EDTA, 0.5 % SDS and 250 µg/ml Proteinase K) (Himedia Laboratories Pvt Ltd., Mumbai, India). RNAse A (Himedia Laboratories Pvt Ltd., Mumbai, India) (0.5 mg/ml) was then added in the lysate and incubation was continued for 1 h. DNA was extracted from the lysate by phenol chloroform method followed by precipitation using cold ethanol in presence of 3 M sodium acetate (pH 5.2). Equivalent amounts of DNA (~20 µg) from different samples were loaded in the wells of 2 % agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) and electrophoresis was done at 50 V for 1 h. DNA was imaged by staining with ethidium bromide using Gel Doc instrument (Uvi Tech).

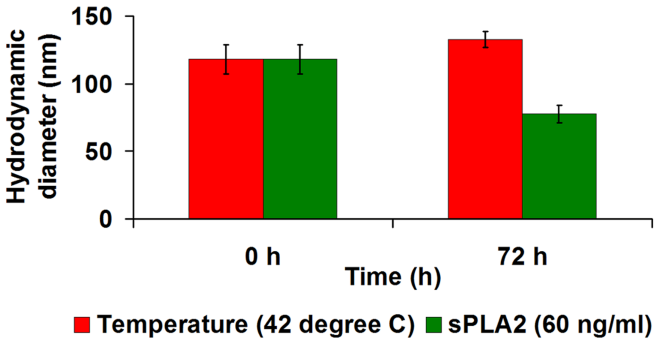

Fig S1. Hydrodynamic diameter of the nanovesicles at 0 h and after 72 h exposure to different triggers (42°C temperature and sPLA2 enzyme) individually.

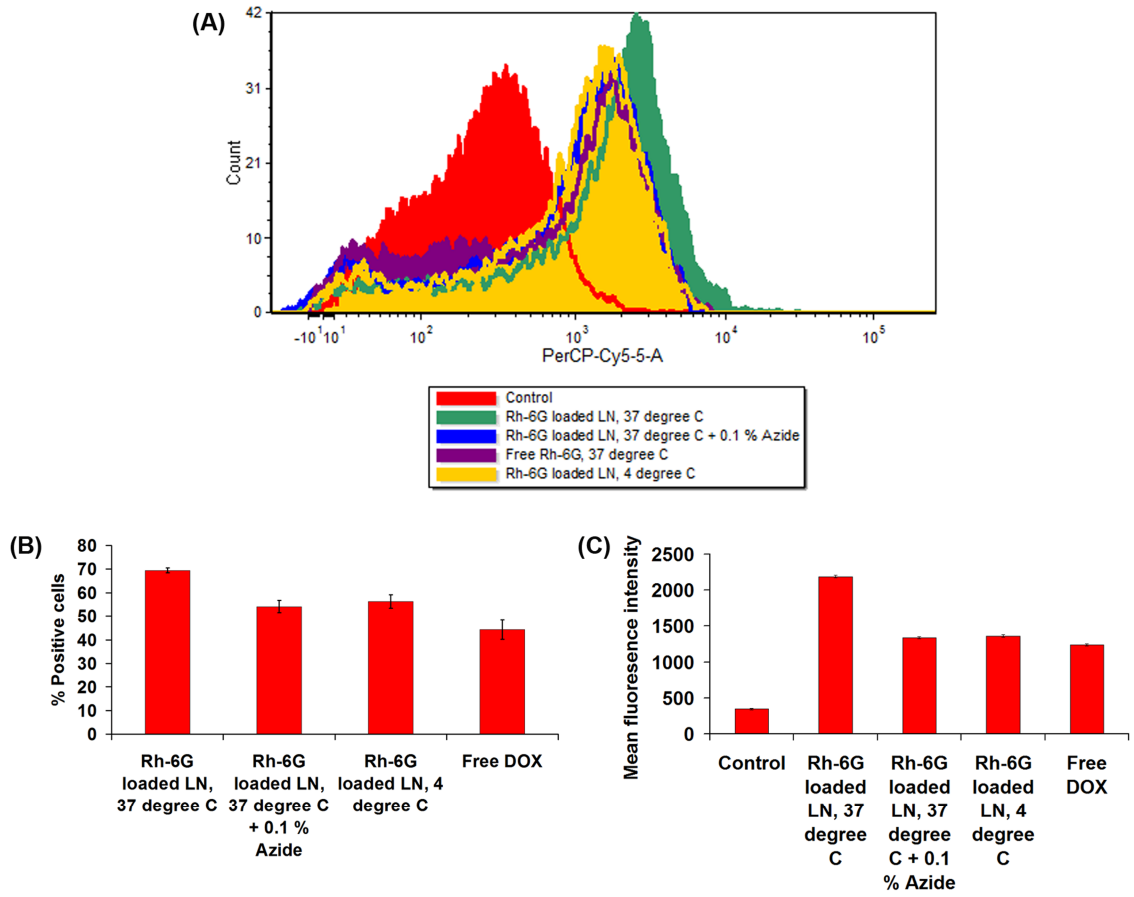

Fig. S2. (A) FACS histograms for cells treated with free Rh-6G or Rh-6G nanovesicles under normal and ATP depleted conditions. **(B)** Percentage positive cells for cells treated with free Rh-6G or Rh-6G nanovesicles under normal and ATP depleted conditions. **(C)** Mean fluorescence intensity of cells treated with free Rh-6G or Rh-6G nanovesicles under normal and ATP depleted conditions.