Inhalable Cationic Niosomes of Curcumin Enhanced Drug Delivery and Apoptosis in Lung Cancer Cells

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ABSTRACT

Background: Curcumin induces apoptosis in non-small cell lung cancer cells. Hence, inhalable cationic niosomes of curcumin were developed to surmount the poor physicochemical and biopharmaceutical limitations for effective drug delivery in lung cancer cells. **Methods:** Curcumin loaded freeze-dried cationic small unilamellar niosomes (Cur-C-SUNS) were prepared using reverse phase evaporation method and characterized *in vitro* using spectral, analytical and biological techniques. **Results:** The nanovesicle size, encapsulation efficiency and zeta-potential of Cur-C-SUNS were measured to be 97.4 ± 8.3 nm, $83.3 \pm 5.1\%$ and $+28.5 \pm 1.25$ mV, significantly ($P < 0.05$) higher than 83.8 ± 7.2 nm, $78.8 \pm 4.5\%$ and -3.02 ± 0.64 mV of optimized freeze-dried Cur-SUNS. Cur-C-SUNS inhibited the A549 lung cancer cells proliferation at the IC_{50} of 3.1 μM, significantly ($P < 0.05$) lower than 7.5 μM of Cur-SUNS and curcumin suspension (< 32 μM). Consistently, Cur-C-SUNS induced greater extent of apoptosis in comparison to Cur-SUNS and curcumin suspension. In addition, Cur-C-SUNS accumulated significantly ($P < 0.05$) higher concentration of curcumin, 14.3 ± 2.1 μg in A549 cells, as compared to 9.5 ± 1.5 μg and 1.3 ± 0.2 μg deposited, respectively by Cur-SUNS and curcumin suspension. At last, *in vitro* cellular uptake illustrated higher endocytosis of Cur-C-SUNS as compared to Cur-SUNS due to electrostatic interaction between cationic nanovesicles and negatively charged plasma membrane of A549 cells. **Conclusion:** In conclusion, promising in vitro attributes of Cur-C-SUNS in lung cancer therapy warrant further *in vivo* tumor regression study to scale up the technology for clinical translation.

Key words: Curcumin, Inhalation, Cationic niosomes, Lung cancer cells, Cytotoxicity, Apoptosis, Cellular uptake.

INTRODUCTION

Curcumin, a major active constituent of *Curcuma longa,* induces apoptosis in non-small cell lung cancer cells including chemoresistant cells in G_2M phase.[1-2] Curcumin augments p53 phosphorylation and activates caspase-3 following *poly (ADP-ribose) polymerase* (PARP) degradation.[3] However, suboptimal therapeutic concentration of curcumin at the site of action hampers pharmacological efficacy that may be attributed to high lipophilicity (log $P \sim 2.92$) and low aqueous solubility (<0.1 mg/ml).[4] Continuation to this, parenteral administration of curcumin at the dose of 100 mg/kg displayed a very short half-life ranging from 3.5 to 6.6 min.[5] Oral administration of 0.4 to 6.0 g of curcumin also demonstrated a very low absolute bioavailability (1%) due to first-pass hepatic metabolism.[6] This entails the administration of multiple doses of curcumin for successive chemotherapy.

Inhalable drug delivery offers several advantages, in comparison to oral and parenteral drug delivery systems.[7] Improved drug delivery, zero-order release and high stability in physiological *milieu* of lungs facilitate a constant bioavailability at the site of action.[8] In this context, inhalable liposomes and niosomes have been extensively investigated for delivery of poorly water soluble drugs.[9-10] Physical and chemical stability of niosomes at accelerated stability testing conditions further enhanced its utility for pulmonary administration via inhalation route of administration.[11] Interestingly, colloidal nanovesicles directly deliver the therapeutic

moiety in the lungs through nasal or intratracheal instillation. However, inhalation route for pulmonary administration is associated with plentiful challenges. Vesicles, ranging from 5-10 μm accumulate in primary bronchi, 1-5 μm particles deposit in secondary bronchi, 1-3 μm size hoards in the bronchioles, and <0.5 to 1 μm retain by alveoli.[12-13] Nanovesicles<100 nm owing to diffusional mobility appear in the alveolar region with a fractional deposition of around 50%. These ultrafine nanovesicles usually enter the lungs as larger agglomerates and consequently segregate into smaller particles on deposition. Furthermore, mucociliary clearance by ciliated epithelial cells limits the extent of drug uptake through inhalation route of administration.[14] Hence, several strategies have been stabbed to enhance the drug stability against mucociliary washout either by the use of permeation enhancers or mucoadhesi vevesiculization.[15-17] In this background, cationic nanovesicles have been widely investigated for delivery of both small and high molecular weight drugs.[18,19] The positive surface charge of cationic nanovesicles allows uptake through inhalation route owing to strong electrostatic interaction between mucus or negatively charged mucosal surface.[20] Therefore, in present investigation, curcumin loaded cationic small unilamellar niosomes (Cur-C-SUNS) were prepared using reverse phase evaporation method[21] and freeze dried. The prepared small unilamellar niosomes were further optimized and characterized for nanovesicle size, shape, surface topography, zeta potential, nanoencapsulation efficiency, *in vitro* drug deposition and release studies. The therapeutic efficacy of freeze-dried Cur-C-SUNS was determined *in vitro* using cell proliferation, intracellular drug concentration, apoptosis and cellular uptake assay carried out in non-small cell lung cancer, A549 cells.

EXPERIMENTAL

Materials

Curcumin [(1E, 6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-Dione)] andspan 80 (Sorbitanmonooleate) were purchased from Loba Chemie, Mumbai, India. Cholesterol was purchased from Nice Chemical Private Limited, Mumbai, India. Cetyl trimethyl ammonium bromide (CTAB) was supplied by Spectrochem Private Limited, Mumbai, India. All other chemicals and solvents used were of highest analytical grade.

Cell culture and reagents

Human non-small lung cancer cell line, A549 was cultured under 95% air and 5% CO_2 atmosphere at 37°C using DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum. All experiments were performed with asynchronous cell populations in exponential growth phase (24 h after plating).[22]

Preparation and optimization of curcumin loaded small unilamellar niosomes

Curcumin loaded small unilamellar niosomes (Cur-SUNS) were prepared by reverse phase evaporation method.[21] In brief, span 80 and cholesterol (μM ratio) were added in different ratio (Table 1) to 5 ml of organic phase (4 ml diethyl ether and 1 ml chloroform) containing 10 mg/ml of curcumin. To this organic phase, 2 ml of phosphate buffer (pH-6.4~nasal *milieu*)[23] was added. The binary phase was sonicated (Tohseon, Mumbai, India) for 10 min at 4°C to form a homogenous emulsion. The emulsion was then vortexed (Shivaki, New Delhi, India) to evaporate the organic solvent. The residual solvent in the emulsion was removed by rotary flash evaporator (E Quivac-V02, Medica Instrument, Mumbai, India) at 60°C under vacuum (250 mm Hg). The emulsion was vortexed again until the gel was collapsed to fluid. To the final solution, 3 ml of phosphate buffer (pH 6.4) was added to hydrate the niosomes. The resulting multilamellar niosomes were passed 5 times through 0.45 μm membrane filter (MDI, Ambala, India) and 5 times through 0.22 μm membrane filter (MDI, Ambala, India) to obtain Cur-SUNS. Cur-C-SUNS were prepared by altering the composition of the optimized formulation (Table 1). The resultant optimized Cur-SUNS and Cur-C-SUNS were freeze-dried separately using mannitol (1 part span 80 and 2 parts mannitol) as a cryoprotectant (Lark India, India).[24] The optimized freeze-dried Cur-SUNS and Cur-C-SUNS were stored in a refrigerator until further use. Blank SUNS and C-SUNS were prepared in a parallel way without incorporation of curcumin.

Characterization of niosomes

Size and zeta potential of nanovesicles

The nanovesicle size and zeta potential of each sample were measured by photon correlation spectroscopy with an autosize IIC apparatus (Malvern Instruments, Worcestershire, UK). The freeze-dried niosomes were reconstituted with phosphate buffer (pH~6.4). Briefly, 100 μL of each nanovesicle sample was diluted with 5 ml of phosphate buffer (pH~6.4) and the mean nanovesicle size was determined. An electric voltage of 150 mV was applied to observe the electrophoretic velocity of nanovesicles. All measurements were made at 25°C in triplicate.

Transmission and scanning electron microscopy (TEM and SEM)

The shape of nanovesicles was examined by Hitachi H 7500 transmission electron microscope at a voltage of 80 kV. The aqueous dispersion of the nanovesicles was drop-cast onto a carbon coated grid and stained with 1% phosphotungustic acid as a negative stain for niosomes. The grid was dried at room temperature before loading it into the microscope. The surface of freeze-dried niosomes was observed with the help of scanning electron microscope. A film of solid samples was prepared separately on aluminum stubs followed by coating with gold to a thickness of 200-500Å under an argon atmosphere. The gold coated samples were then scanned and visualized with SEM (LEO-430; Cambridge, UK).

Nanoencapsulation efficiency

The nanoencapsulation efficiency of each sample was determined by column chromatography technique.[25] In brief, 0.5 ml quantity of each sample was eluted with phosphate buffer (pH~6.4) through a sephadex G-50 minicolumn to separate the drug loaded nanovesicles from unentrapped drug. The column was spun at 10,000 rpm to elute out the niosomes in test tube. Subsequently, 0.5 ml of isopropyl alcohol was added to each eluted niosomal sample to disrupt the vesicles. The liberated curcumin was estimated at 450 nm by using an UV/Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan).[26] All measurements were made at 25°C in triplicate. Nanoencapsulation efficiency (% N.E) was calculated according to Equation (1):

% N.E=Amount of curcumin recovered/Amount of curcumin added × 100 (1)

In vitro drug deposition study

The respirable fine particle fraction of freeze dried niosomes was determined using a twin-stage impinge (TSI), apparatus, as specified in Indian Pharmacopoeia. The optimized freeze-dried Cur-SUNS and Cur-C-SUNS (Table 1) were reconstituted with normal saline and nebulized with the help of a nebulizer attached to the TSI by means of rubber collars. The vacuum pump was operated at 28 L/min. The fractions collected at each step were analyzed for curcumin at 450 nm by using a UV/Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan). Fine particle fraction (FPF) is expressed as the ratio of the amount of drug recovered from the lower stage of the impinger to the sum of drug recovered from the capsule shells, the inhaler device and the upper and lower stages of the twin stage impinger.

In vitro drug release

The *in vitro* drug release of curcumin from customized nanoformulations was measured by dialysis technique.[27] Briefly 2 ml of freeze-dried curcumin suspension (2.5 mg/2 ml), optimized freeze-dried Cur-SUNS (~2.5 mg of curcumin), and freeze-dried Cur-C-SUNS (~2.5 mg of curcumin) were filled separately in dialysis tubes (10 KDa, Sigma, USA). All dialysis tubes were then dipped separately in 300 ml of simulated nasal fluid (SNF, pH~6.4) and simulated lung fluid (SLF, pH~7.2),[28] respectively maintained at 37°C. Dissolution mediums were stirred at 75 rpm, as recommended for dissolution testing of inhalable products.[29] A 5 ml sample was withdrawn from each dissolution medium at different time intervals and replaced with fresh medium to maintain sink conditions. All samples were filtered separately through 0.22 µm membrane filters (MDI, Ambala, India) and absorbance were measured at 450 nm by using a UV-Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan).[26] All measurements were carried out in triplicate.

Therapeutic efficacy testing of curcumin loaded cationic small unilamellar niosomes
In vitro cell proliferation assay

The A549, human non-small cell lung cancer cells were used to assess the cytotoxicity. 6×10^3 A549 cells per well were plated in 200 µL of DMEM medium and incubated for 24 h. After the incubation period, serum DMEM was replaced with serum free-DMEM. Next day, cells were treated with a gradient concentration of 2 to 32 µM of freeze-dried curcumin suspension (suspended in phosphate buffer saline, pH~7.4 and diluted with DMEM), optimized freeze-dried Cur-SUNS (~2 to 32 µM of curcumin), freeze-dried Cur-C-SUNS (~2 to 32 µM of curcumin) and blank SUNS for 72 h. At the end of treatment, 5 mg/ml of MTT was added to each well and the plate was incubated at 37°C in dark for 4 h. The formazan product was dissolved by adding 100 µL of DMSO after removing the medium from each well. The absorbance was read at 560 nm taking 630 nm as the reference wavelength (Tecan, Mannedorf, Switzerland).[30]

Determination of intracellular drug concentration

The intracellular concentration of curcumin in A549 lung cancer cells was determined by high performance liquid chromatography (HPLC) assay.[31,32] Briefly, 2×10^5 A549 cells were treated with ~5 µM of freeze-dried curcumin suspension (PBS, pH~7.4), optimized freeze-dried Cur-SUNS (~5 µM of curcumin) and freeze-dried Cur-C-SUNS (~5 µM of curcumin) for 24 h. At the end

Table 1: Composition and optimization of curcumin loaded small unilamellar niosomes						
Formulation	Span 80	Cholesterol CTAB		Vesicle Size[a]	Zeta-Potential	N.E[a]FPF%[a]
Code	(µM)	(µM)	(µM)	(nM)	(mV)	(%)
Cur-SUNS	9	1	0	55.8 ± 9.6	-15.3 ± 1.6	64.3 ± 4.8	
Cur-SUNS	8	2	0	63.6 ± 8.4	-11.2 ± 2.1	70.2 ± 5.9	
Cur-SUNS (Optimized)	7	3	0	85.3 ± 6.8	-2.91 ± 0.96	80.8 ± 6.8	45.3 ± 2.1
Cur-SUNS	6	4	0	76.9 ± 4.9	-2.12 ± 0.80	72.3 ± 4.8	
Cur-SUNS	5	5	0	71.8 ± 8.6	-1.98 ± 0.24	65.4 ± 3.7	
Cur-C-SUNS	7	2	1	96.2 ± 10.9	+29.8 ± 2.13	85.5 ± 4.5	47.3 ± 2.1
Cur-SUNS (Freeze-dried)	7	3	0	112.8 ± 7.2	-3.02 ± 0.64	78.8 ± 4.5	50.4 ± 1.5
Cur-C-SUNS (Freeze-dried)	7	2	1	120.4 ± 8.3	+28.5 ± 1.25	83.3 ± 5.1	51.3 ± 1.9

Note: Cur-SUNS: Curcumin loaded small unilamellar niosomes, Cur-C-SUNS: Curcumin loaded cationic small unilamellar niosomes, CTAB: Cetyl trimethyl ammonium bromide; N.E: Nano encapsulation efficiency, FPF%: Percent fine particle fraction
[a]All measurements were taken in triplicate (n=3) at 25°C.

of treatment, DMEM was replaced with fresh medium and cells were cultured again for 24 h. After culturing, cells were washed with ice-cold PBS and harvested with 0.025% trypsin and 0.01% EDTA, respectively. Subsequently, treated cells were counted with a haemocytometer and lysed with buffer (Tris 100 mM, EDTA, 5 mM, NaCl 200 mM, SDS 0.2%, pH~8) for 4 h at 55°C to form the homogenates. Protein precipitation was carried out by mixing 0.2 ml of homogenate with 0.6 ml of acetonitrile, which was vortexed (Remi, India) for 10 min followed by centrifugation at 5000 rpm (Thermo Scientific Corporation, USA) for 15 min at 4°C. Supernatants of all samples were filtered separately through 0.22 µm membrane filter (MDI, Ambala, India) and stored at -20°C until further analysis. The experiment was performed in triplicate.

The HPLC system was consisted of ELICO HP 464 pump from ELICO (Hyderabad, India) equipped with ELICO HD 469 UV-Vis detector. Chromatographic separation was achieved on a 5 µm particle size packed in reverse phase C18 column (4.6×150 mm). The mobile phase consisted of acetonitrile: 5% acetic acid (75:25% v/v) and pH was adjusted to 3.0. Mobile phase was pumped through the column at the flow rate of 1.0 ml/min. The injection volume was 20 µL. Curcumin was analyzed at 254 nm.[32]

Determination of extent of apoptosis

The extent of apoptosis was determined by commercial fluorometric caspase-3 apoptosis assaykit.[33] In brief, $1×10^5$ A549 cells were treated with ~5 µM of freeze-dried curcumin suspension (PBS, pH~7.4), optimized freeze-dried Cur-SUNS (~5 µM of curcumin) and

freeze-dried Cur-C20 SUNS (~5 µM of curcumin) for 48 h. At the end of treatment, cells were collected in a pellet form and resuspended in 50 µM of cell lysis buffer, followed by incubation on ice for 10 min. Subsequently, cells were centrifuged at 3000×g for 5 min, and the supernatant was transferred to a 96-wells microtitre plate. To this plate, 50 µL of reaction buffer (50 mM/L PIPES, pH~7.4, 10 mM/L EDTA, 0.5% CHAPS) containing 10 mM/L dithiothreitol and 5 µL of the respective substrate was added. The plate was incubated at room temperature for 1 h, and the fluorescence was measured in a fluorometer (exe~400 nm, ~emi 505 nm). The protein content was measured using Bradford assay method.[34] The experiment was performed in triplicate (n=3).

In vitro quantitative and qualitative cellular uptake assay

The A549 cells were cultured in Lab-Tek II Chamber Slide™ System (NalgeNune, USA) at a density of $6×10^3$ cells per chamber. Subsequently, optimized freeze-dried Cur-SUNS and freeze dried Cur-C-SUNS at the concentration ~2 to 32 µM of curcumin were added to phosphate buffer saline (PBS, pH~7.4) and diluted with DMEM to prepare the dosing solutions. Cells were rinsed thrice and pre-incubated with 1 ml of DMEM at 37°C for 1 h. At last, 1 ml of the specified dosing solution was exchanged with DMEM, followed by incubation of the cells at 37°C for 24 h. The experiment was ended up by washing the cells thrice with ice-cold PBS (pH~7.4) and lysed with 1 ml of 0.5% Triton X-100. Cells associated curcumin fluorescence was quantified by analyzing the cell lysate in a fluorometer (Spectra Fluor, Tecan, Switzerland, λexe~553 nm, λemi~574 nm).[35] The protein

content of the cell lysate was measured using Bradford assay method.[34] After treating the A549 cells with niosomes, the medium was removed and plates were washed thrice with sterile PBS (pH~7.4). Ultimately cells were fixed with 4% paraformaldehyde and individual cover slips were mounted on clean glass slides with fluoromount-G mounting medium (Southern Biotechnology, Birmingham, AL). The slides were viewed under confocal laser scanning microscope at λexe~553 nm and λemi~574 nm[35] to capture the images. DAPI (4,6-diamidino-2-phenylindole) was used for nucleus staining.

Statistical analysis

Results are expressed as mean $\pm$ S.D (n=3). Graph 1 Pad Prism 4.0 software was used to calculate the significant difference between mean values using Unpaired 't' test, one and two ANOVA tests. $P<0.05$ value was considered for significant difference.

RESULTS AND DISCUSSION
Preparation and characterization of curcumin loaded cationic small unilamellar niosomes

Oral and parenteral delivery of curcumin for the treatment of non-small cell lung cancer are suffered with extensive first pass metabolism and erratic bio-distribution resulting in low and variable bioavailability at the site of action.[6,36] Inhalation route is promising for delivery of nanotherapeutics in the treatment of non-small cell lung cancer.[37,38] In this context, unilamellar vesicular drug delivery systems have been proven useful for deep lung deposition of chemotherapeutic drugs.[39] Small unilamellar nanovesicles $\leq$0.1 µm are less susceptible to opsonization as compared to large vesicles (>0.1 µm) and thus exhibited longer circulating half life. Furthermore, in post-nebulization, small unilamellar vesicles were examined to be less leaky than large multilamellar vesicles.[40] Hence, the preferred nanovesicle size for clinical application was optimized to be 50-200 nm in diameter.[41] Therefore, in present investigation, curcumin loaded small unilamellar niosomes (Cur-SUNS) and cationic small unilamellar niosomes (Cur-C-SUNS) were prepared by reverse phase evaporation method[21] with slight modification in solvent system. A mixture of diethyl ether and chloroform was used as the organic phase to solubilize the curcumin. Cetyltrimethyl ammonium bromide (CTAB) was included in the optimized Cur-SUNS (Table 1) to impart the cationic charge. CTAB is a biodegradable cationic surfactant which is highly implicated for transfection of nanotherapeutics in cancer cells.[42,43] Furthermore, reverse phase evaporation

method, opted for preparation of niosomes is associated with high encapsulation efficiency of therapeutic molecules.[44] Next, freeze-drying is a potential method to impart the long term stability to nanovesicles. In fact, freeze-drying prevents hydrolysis of the surfactant and thus ensures chemical and physical stability. Hence, optimized Cur-SUNS and Cur-C-SUNS were freeze dried using mannitol as a cryoprotectant. In addition, mannitol, being a mucolytic agent[45] increases the mucus clearance and protects the hydration and surface properties of nanovesicles. Next, Cur-SUNS and Cur-C-SUNS were characterized for vesicles size, zeta potential, and nanoencapsulation efficiency to optimize the nanoformulations for augmenting the cellular uptake in nonsmall cell lung cancer, A549 cells. The mean nanovesicle size of Cur-SUNS was increased significantly (One way ANOVA test, $P<0.05$) from 55.8 $\pm$ 9.6 nm to 85.3 $\pm$ 6.8, as a function of cholesterol content (Table 1). This may be attributed to the critical cholesterol packing parameter,[46] which was reached to a plateau in 7:3 ratios. However, further enhancement in cholesterol content did not increase the nanovesicle size. Hence, optimum span 80: cholesterol ratio was investigated to be 7:3 µM with maximum percent nanoencapsulation efficiency of 80.8 $\pm$ 6.8% and designated as optimized Cur-SUNS. The higher nanoencapsulation efficiency in optimized Cur-SUNS may be contributed to well pack bimolecular film formation. In addition, cholesterol is also known to abolish the gel to liquid phase transition of the niosomes, resulting in a less leaky structure.[21] The mean vesicle size of optimized Cur-SUNS (85.3 $\pm$ 6.8 nm) was significantly (Unpaired 't' test, $P<0.05$) lesser than 96.2 $\pm$ 10.9 nm of Cur-C-SUNS (Table 1). Alteration in optimized Cur-SUNS with CTAB greatly influenced the critical packing parameter of cholesterol and consequently changed the geometry of liquid crystalline state and enhanced the vesicle size. Moreover, owing to loosely held crystalline geometry, nanoencapsulation efficiency was also significantly (Unpaired 't' test, $P>0.05$) enhanced in Cur-C-SUNS (85.5 $\pm$ 4.5%) as compared to Cur-SUNS (80.8 $\pm$ 6.8%). The zeta potential of Cur-C-SUNS was measured to be +29.8 $\pm$ 2.13 mV that was significantly (Unpaired 't' test, $P<0.05$) higher than -2.91 $\pm$ 0.96 mV of Cur-SUNS (Figure 1a-b and Table 1). However, there was no remarkable change in mean vesicle size, zeta potential and nanoencapsulation efficiency of optimized Cur-SUNS and Cur-C-SUNS after freeze drying (Table 1). This substantiated that mannitol retained the original attributes of niosomes upon reconstitution of freeze dried formulations in physiological medium. The shape and surface morphology of niosomes was visualized with TEM and SEM (Figure 1a-b).

The photomicrographs conferred sub nanometer resolution of both Cur-SUNS and Cur-C-SUNS before and after freeze-drying. Niosomal vesicles were appeared as spherical and unilamellar. Moreover, no structural deformation was observed after freeze drying thus promising the stability of respirable formulations. The FPF of both optimized Cur-SUNS and Cur-C-SUNS before and after freeze drying was ranged around 50%, indicating good respirable properties (Table 1). Previous literature review indicated that pulmonary administration of cationic nanovesicles elicited dose-dependent toxicity and inflammation at the site of action.[47,48] Hence, it is necessary to keep the concentration of cationic surfactant below toxic level, while designing the nanoformulation. Our data indicated that freeze-dried Cur-C-SUNS is a stable, reproducible nanoformulation and may be a good candidate for testing the therapeutic efficacy against A549, non-small cell lung cancer cells. A549 cell line represents the alveolar type II pulmonary epithelial cells which has endocytic ability and exhibits the expression of cytochrome P450.[49] Hence A549 cell line is an ideal model to investigate the mechanism of uptake of drug delivery systems, intended for alveolar pulmonary epithelium.

In vitro drug release

In vitro release of curcumin form nanoformulations was measured using dialysis membrane technique.[27] The percent release of curcumin from freeze-dried curcumin suspension, optimized freeze-dried Cur-SUNS and freeze-dried Cur-C-SUNS was measured to be 10.1 ± 1.9%, 22.5 ± 2.1% and 30.1 ± 4.5%, respectively in SNF (pH~6.4) in 3 h (Figure 2a). Continuation to this, freeze-dried curcumin suspension, optimized freeze-dried Cur-C-SUNS and freeze-dried Cur-SUNS released significantly (One-way ANOVA, *P*<0.05) higher 16.1 ± 2.4%, 94.3 ± 2.5% and 87.4 ± 1.2% of curcumin in SLF (pH~7.2) in 72 h in comparison to SNF (pH~6.4) (Figure 2b). Hence, curcumin exhibits different dissolution profile at pH 6.4 and pH 7.2. This may be contributed to the fact that curcumin exhibits pH dependent solubility and highly soluble at alkaline pH.[50] Hence, suppression of release rate from curcumin suspension was noticed at both nasal and lungs pH owing to low solubility and high lipophilicity.[4] On the other hand, release rate was improved from both freeze dried Cur-SUNS and Cur-C-SUNS due to amorphization of nanovesicles that ultimately enhanced the solubility and release rate.[51] Moreover, it is also documented that freeze drying is good technique to improve the long term stability of colloidal nanovesicles.[52] Hence, freeze-dried Cur-C-SUNS may be suitable for inhalation route of administration.

### Cur-C-SUNS potentially inhibited A549 cell proliferation

The freeze-dried Cur-C-SUNS inhibited the A549 lung cancer cells proliferation at the IC$_{50}$ of 3.1 μM, significantly (One way ANOVA test, *P*<0.05) lower than 7.5 μM of optimized freeze-dried Cur-SUNS and <32 μM of freeze-dried curcumin suspension (Figure 3a). Although cationic charge appears to enhance the efficacy of cellular imaging, gene delivery, and drug delivery, a higher cytotoxicity of such delivery systems has been reported.[53] Cationic NPs promotes more impactful disruption of plasma-membrane integrity, stronger mitochondrial and lysosomal damage than negatively charged nanovesicles.[54,55] Hence, Cur-C-SUNS owing to strong electrostatic interaction with negatively charged cell membrane of cancer cells[56] promoted the diffusion of curcumin to a higher extent as compared to Cur-SUNS. In contrast, curcumin suspension did not endorse the transportation of drug across the cellular membrane due to flocculation.[57] Blank SUNS did not induce any toxicity in A549 lung cancer cells (Data not shown).

Cur-C-SUNS augmented intracellular drug concentration in A549 cells

Consistently, freeze-dried Cur-C-SUNS induced significantly (One-way ANOVA test, *P*<0.05) higher concentration, 14.3 ± 2.1 μg in A549 cells, as compared to 9.5 ± 1.5 μg by optimized freeze dried Cur-SUNS and negligible amount, 1.3 ± 0.2 μg by freeze-dried curcumin suspension (Figure 3b). This could be credited to higher intracellular penetration of cationic nanovesicles in A549 lung cancer cells. The lipid bilayer architecture of the cell membrane along with integrated proteins separates cell from their external *milieu*. Hence, cationic nanovesicles penetrated the negatively charged surface of cancer cells and endocytosed by cell membrane transport machinery, such as non-receptor mediated endocytosis mechanism more efficiently than anionic or neutral vesicles.[58]

### Cur-C-SUNS exhibited enhanced degree of apoptosis

The extent of apoptosis was measured by Caspase-3 cleavage and expressed in terms of mean fluorescence intensity. The freeze-dried Cur-C-SUNS induced significantly (One-way ANOV test, *P*<0.05) a greater degree of apoptosis as compared to optimized freeze-dried Cur-SUNS and freeze-dried curcumin suspension (Figure 3c). The greater degree of apoptosis induced by freeze dried Cur-C-SUNS may be attributed to augmentation of intracellular drug concentration in A549 cells. Blank SUNS and C-SUNS did not initiate the apoptosis remarkably in A549 cells.
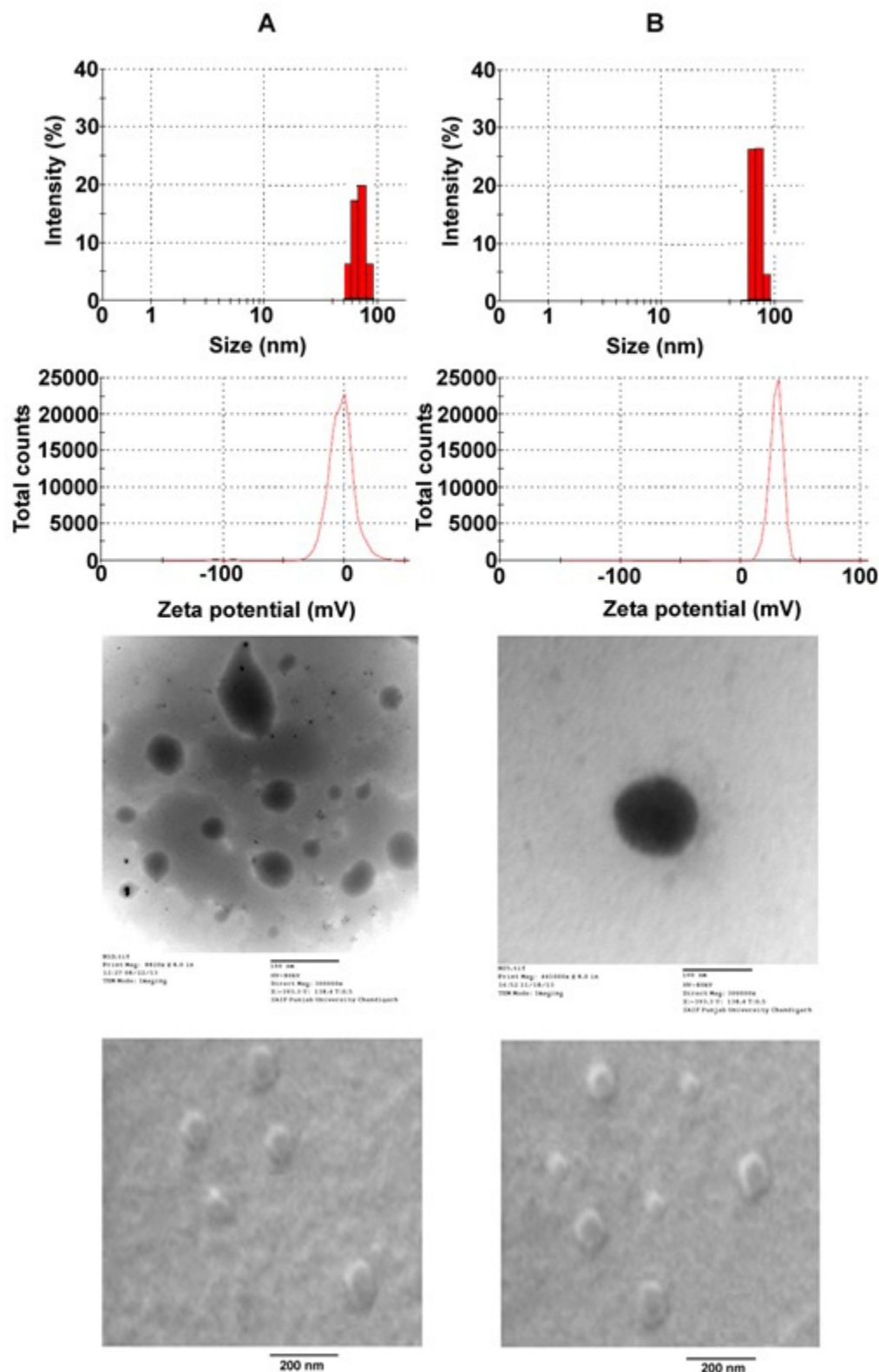
Figure 1: Nanovesicle size, zeta potential, transmission and scanning electron microscopy of A) Optimized curcumin loaded small unilamellar niosomes, Cur-SUNS and; B) Curcumin loaded cationic small unilamellar niosomes, Cur-C-SUNS. The mean nanovesicle size and zeta potential of Cur-SUNS before freeze drying were measured to be 85.3 ± 6.8 nm/-2.91 ± 0.96 mV significantly (Unpaired 't' test, *P*<0.05) lower than 96.2 ± 10.9 nm/+29.8 ± 2.13 mV of Cur-C-SUNS. Scale bar~100 nm (TEM). Scanning electron microscopy was performed for freeze-dried niosomes at scale bar~200 nm.

Each experiment was performed in triplicate.
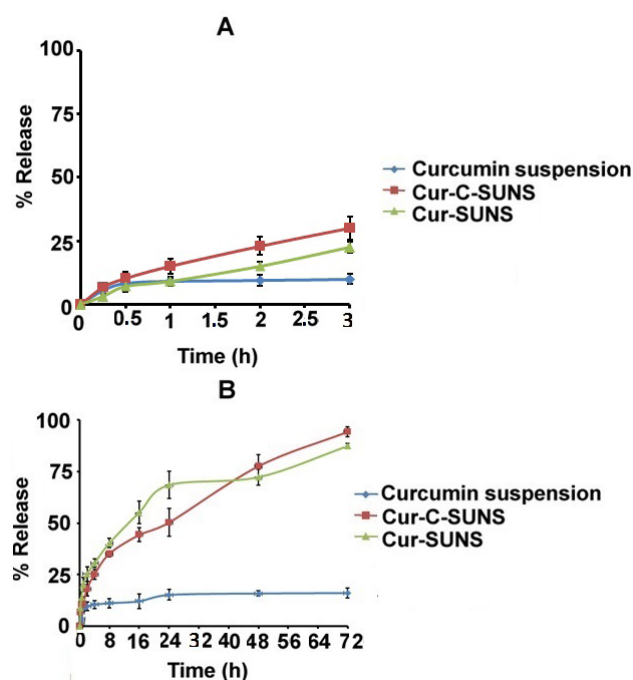
Figure 2: *In vitro* release of curumin from niosomes in A) Simulated nasal fluid (SNF, pH~6.4), and; B) Simulated lung fluid (SLF, pH~7.4). The percent release of curcumin from freeze-dried curcumin suspension, optimized freeze-dried Cur-SUNS and freeze-dried Cur-C-SUNS was measured to be 10.1 ± 1.9%, 22.5 ± 2.1% and 30.1 ± 4.5%, respectively in SNF (pH~6.4) in 3 h. Furthermore, freeze-dried curcumin suspension, optimized freeze-dried Cur-C-SUNS and freeze dried Cur-SUNS released significantly (One-way ANOVA, P<0.05) higher 16.1 ± 2.4%, 94.3 ± 2.5% and 87.4 ± 1.2% of curcumin in SLF (pH~7.2) in 72 h in comparison to SNF (pH~6.4).

Each experiment was performed in triplicate.

## Cur-C-SUNS and Cur-SUNS exhibited concentration dependent cellular uptake

The quantitative cellular uptake in A549 cells indicated the concentration dependent internalization of both freeze-dried Cur-C-SUNS and optimized freeze-dried Cur-SUNS, as measured by fluorometry. Cur-C-SUNS exhibited significantly (Unpaired '*t*' test) higher 56.3 ± 2.4% of cellular uptake, as compared to 44.1 ± 4.8% by Cur-SUNS (Figure 4a). Moreover, a representative confocal laser scanning microscope (CLSM) image showed that freeze-dried Cur-C-SUNS were internalized more intensely as compared to optimized freeze-dried Cur-SUNS by A549 cells (Figure 4b). We propose that Cur-C-SUNS might be internalized due to surface interaction of negatively charged plasma membrane of A549 cells and positively charged cationic nanovesicles via clevaloe mediated/independent endocytosis thus randomly distributed in the cytoplasm by endosomal-lysosomal escape mechanism. In addition, particle size of cationic nanovesicles also played a crucial role in recognition and internalization, as it is reported that
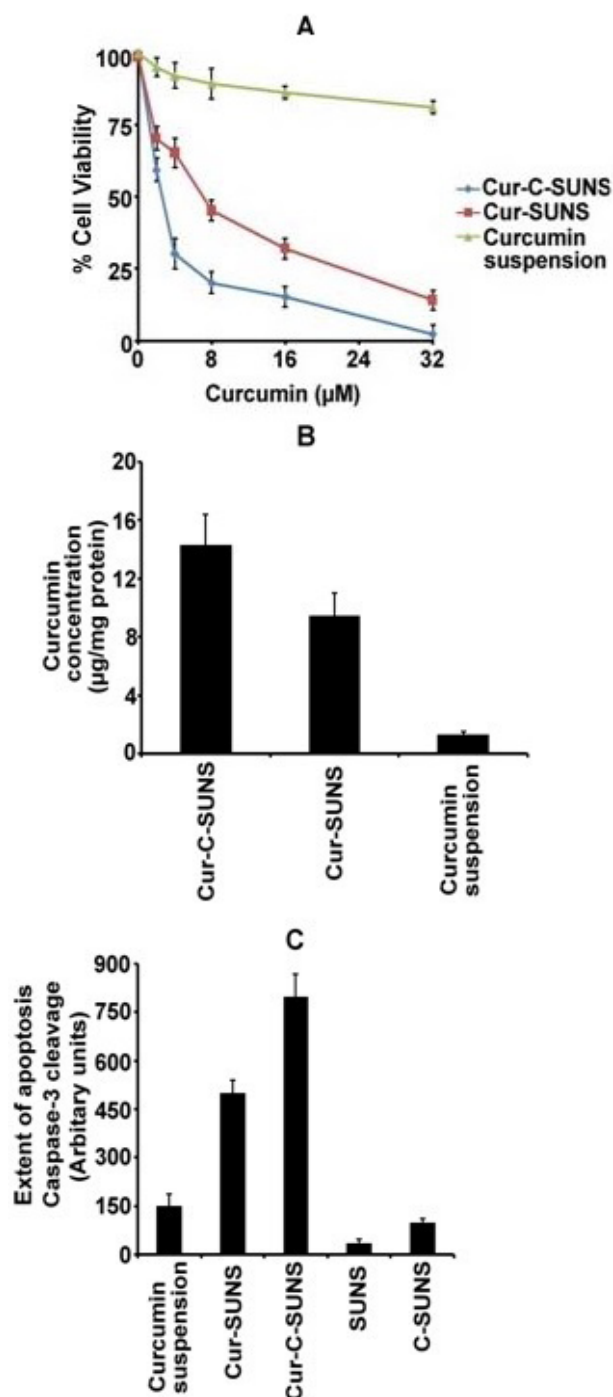
Figure 3: A) *In vitro* cell proliferation assay performed in A549, non-small cell lung cancer cells. The IC$_{50}$ was observed to be in following order: 3.1 µM>7.5 µM and <32.0 µM for freeze-dried Cur-C-SUNS, optimized freeze-dried Cur-SUNS and freeze-dried curcumin suspension, respectively; B) Intracellular drug concentration was measured to be 14.3 ± 2.1µg, 9.5 ± 1.5 µg and 1.3 ± 0.2 µg/mg of protein, respectively for freeze-dried Cur-C-SUNS, optimized freeze-dried Cur-SUNS and freeze-dried curcumin suspension in A549 cells and; C) Extent of apoptosis expressed in terms of caspase-3 cleavage (arbitrary units). Higher degree of apoptosis was induced by freeze-dried Cur-C-SUNS followed by optimized freeze-dried Cur-SUNS and freeze dried curcumin suspension.

Each experiment was performed in triplicate.
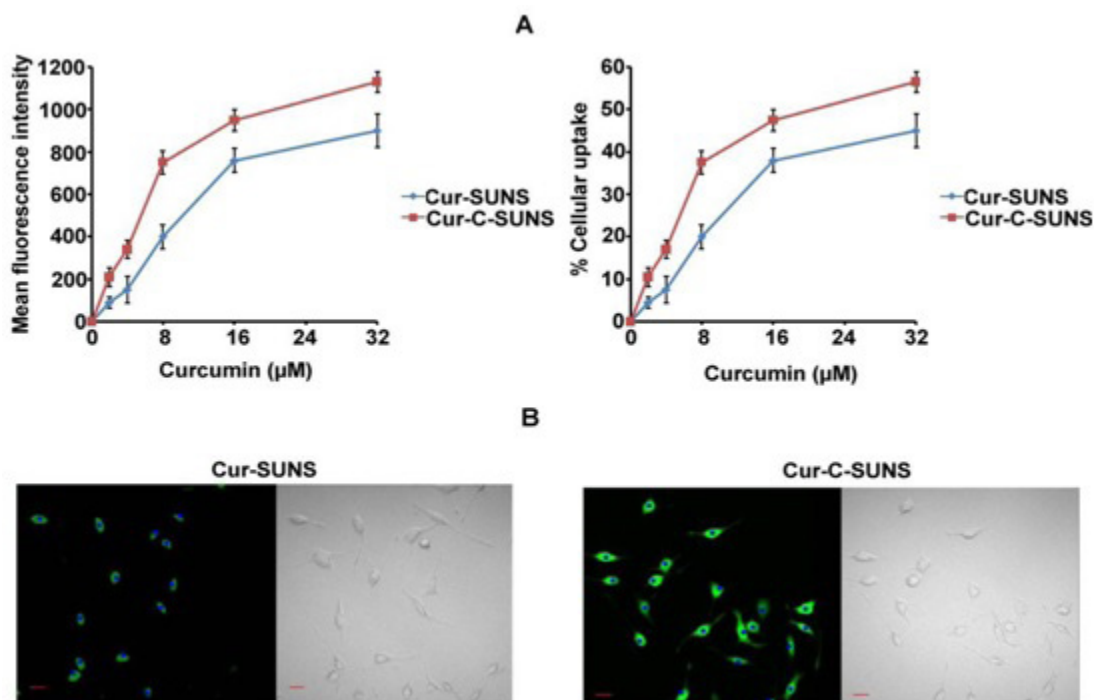
Figure 4: Cellular uptake assay expressed quantitatively as A) Mean fluorescence intensity vis-à-vis freeze dried curcuin loaded niosomes; % cellular uptake vis-à-vis freeze dried curcumin loaded niosomes. Freeze-dried Cur-C-SUNS exhibited significantly (Unpaired 't' test) higher 56.3 ± 2.4% of cellular uptake, as compared to 44.1 ± 4.8% by optimized freeze-dried Cur-SUNS and; B) Confocal laser scanning microscopy (CLSM) of cellular uptake of optimized freeze-dried Cur-SUNS and freeze-dried Cur-C-SUNS in A549, non-small cell lung cancer cells. DAPI was used for nucleus staining.
Each experiment was performed in triplicate.

particles up to 500 nm sizes were taken up by A549 cells more efficiently than particles of 10 µm.[53,59] In addition, the *in vitro* assays demonstrated that cationic nano-vesicles may be a safe and efficacious inhalable dosage form for *in vivo* delivery of chemotherapeutic moiety. Moreover, inhalable carrier delivery device must aerosol-ize the drug in the appropriate particle size distribution and concentration to ensure optimal deposition and dose in the desired region of the lung.

CONCLUSION

Our studies displayed that nanoformulation, Cur-C-SUNS bear suitable nanovesicle size for inhalation with good drug entrapment efficiency. The *in vitro* release studies from freeze-dried Cur-C-SUNS demonstrated that cur-cumin was released in a sustained release manner over a prolonged period. Intracellular uptake and cell viability assays also demonstrated efficient internalization and significant cytotoxicity in A549 non-small cell lung cancer cells. In conclusion, freeze-dried Cur-C-SUNS intended for inhalation route of administration are capable to deliver high therapeutic concentration of curcumin over a prolonged period in physiological *milieu* of lungs. Thus, freeze-dried Cur-C-SUNS may be a

promising inhalable nanomedicine and warrant further in-depth *in vitro* and *in vivo* investigations to scale-up the technology for clinical translation.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

There is no conflicts of interest.

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PICTORIAL ABSTRACT
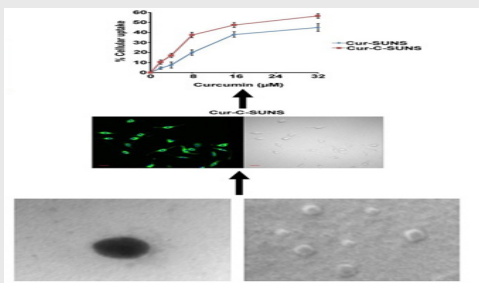
SUMMARY

- Curcumin induces apoptosis and cell death in non-small cell lung cancer cells
- Inhalable cationic niosomes surmounted the physicochemical limitations of curcumin
- Inhalable cationic niosomes of curcumin improved drug delivery in lung cancer, A549 cells

ABBREVIATIONS USED

Cur: Curcumin; **SUNS:** Small unilamellar niosomes; **DMEM:** Dulbecco's Modified Eagles Medium; **TEM:** Transmission electron microscopy; **SEM:** Scanning electron microscopy; **SNF:** Simulated nasal fluid; **SLF:** Simulated lungs fluid; **DMSO:** Dimethyl sulfoxide, **HPLC:** High performance liquid chromatography.

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