Comparative study of transfersomes, liposomes, and niosomes for topical delivery of 5-fluorouracil to skin cancer cells: preparation, characterization, in-vitro release, and cytotoxicity analysis

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Topical 5-fluorouracil (5-FU) is used for the treatment of actinic keratosis and nonmelanoma skin cancer. Unfortunately, 5-FU *per se* shows a poor percutaneous permeation, thus reducing its anticancer effectiveness after topical administration. Therefore, we have constructed transfersomes, liposomes, and niosomes of 5-FU for topical applications in this investigation. Transfersomes were prepared by the solvent evaporation method, whereas liposomes and niosomes were constructed by reverse-phase evaporation method. The nanovesicles were characterized for particle size, shape, zeta potential, viscosity, entrapment efficiency, deformability, in-vitro permeation release, and kinetics and retention. Cytotoxicity study was carried out on HaCaT cells. Transfersomes (153.2 ± 10.3 nm), liposomes (120.3 ± 9.8 nm), and niosomes (250.4 ± 8.6 nm) were produced with a maximum entrapment efficiency of 82.4 ± 4.8, 45.4 ± 3.3, and 43.4 ± 3.2%, respectively. Moreover, transmission electron microscopy and atomic force microscopy assure the smooth and spherical shape of nanovesicles. Skin permeation and retention showed better permeability and retention than the nonvesiculized dosage form. The IC_{50} value of transfersomes (1.02 μmol/l), liposomes (6.83 μmol/l), and niosomes (9.91 μmol/l) was found to be far less than 5-FU (15.89 μmol/l) at 72 h. 5-FU-loaded transfersomes were found to be most cytotoxic on the HaCaT cell line in comparison with liposomes and niosomes. We concluded that vesiculization of 5-FU not only improves the topical delivery, but also enhances the cytotoxic effect of 5-FU. We have presented here a viable formulation of 5-FU for the management of actinic keratosis and nonmelanoma skin carcinoma. *Anti-Cancer Drugs* 22:774–782 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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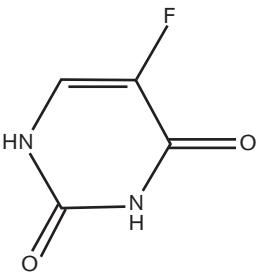
Introduction

5-Fluorouracil (5-FU), a hydrophilic pyrimidine analog, causes cell death by interfering with DNA synthesis, and is widely used in various forms of skin cancers (Fig. 1) [1,2]. This active compound showed a suitable anticancer effect in the topical treatment of lesions related to squamous cell carcinoma such as actinic keratosis, Bowen's disease, and keratoacanthoma [3]. Moreover, therapy for basal and squamous cell carcinoma does not end with the treatment of the initial lesion because almost 50% of patients with one nonmelanoma skin cancer develop another one within the next 5 years [4]. Therefore, an improved percutaneous permeation of 5-FU is a fundamental requisite to achieve an effective topical therapeutic approach. Unfortunately, 5-FU *per se* shows a poor percutaneous permeation, thus reducing its anticancer effectiveness after topical administration [5,6]. To conquer these shortcomings, liposomes and niosomes have been reported for topical delivery of 5-FU in skin cancer [7,8]. However, transfersomes that are elastic nanovesicles, composed of phosphatidylcholine and cholate, differ from conventional liposomes and niosomes by their characteristic fluid membrane with high elasticity. This feature enables transfersomes to squeeze themselves through the intercellular regions of stratum corneum under the

Fig. 1



Schematic representation of the chemical structure of 5-fluorouracil.

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influence of a transdermal water gradient [9]. In contrast, liposomes/niosomes are microscopic vesicles composed of one or more lipid/nonionic surfactant bilayers arranged in a concentric manner enclosing an equal number of aqueous compartments [10]. Moreover, niosomes have more chemical stability than liposomes, but they are less deformable than liposomes and transfersomes [11]. Recently, we have reported polymeric nanovesicles for intracellular delivery of anticancer drug to tumor network [12,13]. Therefore, in this investigation, we have focused on the construction of 5-FU nanovesicles, namely transfersomes, liposomes, and niosomes, and a comparative study was conducted for permeability, release kinetics, retention, and in-vitro cytotoxicity. In-vitro cytotoxicity study was carried out on the HaCaT (nonmelanoma skin cancer) cell line that present an early stage of skin cancer [14].

Materials and methods

Materials

5-FU was obtained as gift sample from Shalaks Pharmaceuticals, New Delhi, India. Soya phosphatidylcholine (SPC), cholesterol, sodium deoxycholate (SDC), and Sephadex G-150 were purchased from Sigma Aldrich, St Louis, Missouri, USA. Carbopol 941 was purchased from CDH, New Delhi, India. Span 80 was obtained from Loba Chemie, Mumbai, India. All other chemicals used were of analytical grade.

Preparation of transfersomes

5-FU-loaded transfersomes (5-FUTR) were prepared by the solvent evaporation method [15]. In brief, an ethanolic solution of SPC (% w/w) was mixed with SDC (% w/w) in a round-bottom flask. Subsequently, the organic solvent was removed using a rotary evaporator (Buchi, CH-9230, Flawil 1, Switzerland) under reduced pressure at 40°C. Final traces of solvent were removed under vacuum overnight. The deposited film was hydrated with phosphate buffer (pH: 6.5) containing 5-FU solution (10 mg/ml). The obtained suspension was pushed 10 times through a series of 0.45 and 0.22 μm polycarbonate membranes (MDI, Ambala, India).

Preparations of liposomes and niosomes

5-FU-loaded liposomes (5-FULS) and niosomes (5-FUNS) were prepared by a reverse-phase evaporation technique [16,17]. In brief, liposomes were fabricated by dissolving SPC and cholesterol (mol/l) in 5 ml of diethyl ether (organic phase) in a glass boiling tube, to which 2 ml of aqueous phase, phosphate buffer-containing 5-FU (pH 6.5; 10 mg/ml) was added. The mixture was sonicated (Tohseon, Mumbai, India) for 5 min at 4°C and was attached to a rotary evaporator to dry the contents at 37°C under vacuum (250 mmHg) until a gel was formed. Vacuum was released and the tube was removed from the evaporator and subjected to vigorous mechanical agitation on a vortex mixer (Shivaki, New Delhi, India) for 5 min. When the gel collapsed to fluid, it was again fitted to the rotary flash evaporator for the removal of the organic solvent. The final liposomal suspension was subjected to complete removal of the last traces of organic solvent in the rotary flash evaporator under vacuum (600 mmHg) for 15 min. To this emulsion, 2 ml of phosphate buffer (pH 6.5) was added to hydrate the mixture, resulting in the formation of large unilammelar vesicles. The resulting large unilammelar vesicles were passed five times through 0.45 and 10 times through 0.22-μm polycarbonate membranes (MDI). A similar method was adopted for the fabrication of 5-FUNS.

Vesicular size

The particle size of 5-FUTR, 5-FULS, and 5-FUNS was measured by photon correlation spectroscopy with an autosize IIC apparatus (Malvern Instruments, Worcestershire, UK). For particle size analysis, 100 μl of vesicular suspension was dispersed separately in 4 ml of phosphate buffer (pH 6.5), and the mean particle size was determined. All the measurements were taken at 25°C in triplicate.

Zeta potential

Zeta potential of 5-FUTR3, 5-FULS3, and 5-FUNS3 vis-à-vis blank formulations of the same (BTR3, BLS3, and BNS3) dispersed in phosphate buffer (pH 6.5) separately was determined by laser Doppler anemometry using a Zetasizer (Malvern Instruments, UK). For the measurement, 100 μl of the respective nanovesicle suspension was diluted to 4 ml with phosphate buffer (pH 6.5) and an electric field of 150 mV was applied to observe the electrophoretic velocity of the vesicles. All measurements were made at 25°C in triplicate in the same ionic concentration.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to examine the shape and morphology of nanovesicles using 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 transfersomes and liposomes. The grid was dried at room temperature before loading it into the microscope.

Atomic force microscopy

The shape and surface morphology of the nanovesicles were also investigated using atomic force microscopy (AFM; Agilent Technology, Santa Clara, California, USA). Imaging was done in air using a 0.7-μm AFM head. The sample is placed on a xyz-piezo-translator and scanned using a sharp diamond tip mounted on a gold-coated 200-μm triangular Si_3N_4-microfabricated cantilever (force constant = 0.6 N/m). The force between the tip and the sample usually ranges from 10^{-7} to 10^{-9} N.

Encapsulation efficiency

A volume of 0.2 ml of 5-FUTR, 5-FULS, and 5-FUNS was eluted with phosphate buffer (pH: 6.5) through a Sephadex G-150 minicolumn to separate the unentrapped 5-FU [17]. The method was repeated three times with a fresh syringe packed with gel each time. The fraction was finally collected free from unentrapped 5-FU. To the vesicular fraction, 0.5 ml of isopropyl alcohol was added to disrupt the vesicles. The liberated 5-FU was estimated by a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) at 266 nm [18].

Deformability index

Comparative measurement of elasticity of the bilayer of 5-FUTR3, 5-FULS3, and 5-FUNS3 was carried out by extrusion measurement [19]. In brief, the vesicles were extruded through a polycarbonate filter with a pore size of 50 nm (MDI) at constant pressure. The elasticity of the vesicle was expressed as:

$$\text{Deformability index} = J(r_v / r_p)^2.$$

where j is the weight of the suspension, which was extruded in 10 min through a polycarbonate filter of 50-nm pore size, r_v is the size of the vesicle, and r_p is the pore size of the membrane.

Storage-stability studies

The ability of nanovesicles to retain the drug was assessed by keeping transfersomes, liposomes, and niosomes at three different temperature conditions, that is, 4–8°C (refrigerator), 25 ± 2°C (room temperature), and 37 ± 2°C for a period of 5 weeks. The nanovesicle suspensions were kept in sealed ampoules (10-ml capacity) after nitrogen flushing. Samples were withdrawn periodically and analyzed for particle size and drug content.

Preparation of 5-fluorouracil nanovesicle gel

Carbopol gel (0.5% w/v) was prepared in water and gently stirred for the gel to swell. A volume of 5 ml of triethanolamine and water were alternatively added with continuous stirring to form a transparent gel. Optimized formulation of nanovesicles, that is, 5-FUTR3, 5-FULS3, and 5-FUNS3 were transferred to the carbopol gel to fabricate 5-FU nanovesicle gel. Similarly, 5-FU-bearing carbopol gel was prepared as the control.

Measurement of rheological properties

The viscosity of optimized batches (5-FUTR3, 5-FULS3, and 5-FUNS3) incorporated into the gel was determined and compared with the 5-FU gel at different shear rates. The viscosity was determined using a HAAKE Visco-Tester 550 (rotational viscometer; Thermo Scientific, Mumbai, India) and viscosity was calculated with special HAAKE RheoWin software (Thermo Scientific) and expressed as Pa/s (kg/m/s).

In-vitro skin permeation and release kinetic study

Skin permeation and release kinetic study of optimized batches (5-FUTR3, 5-FULS3, and 5-FUNS3) incorporated nanovesicle gel, aqueous solution of 5-FU (5-FUAQ) and 5-FU gel was carried out using Franz diffusion cell mounted with abdominal skin of male rats [20]. The study was conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals, Ministry of Culture, Government of India. The Institutional Animal Ethics Committee approved the study. To conduct the study, rats were killed and hairs on the dorsal side of the rat were removed using a hair-removal cream. Dermal part of the skin was washed thoroughly with 0.9% NaCl and wiped with cotton swab wetted with isopropanol to remove the remaining fat material, if any. Skin was mounted on the receptor chamber with a cross-sectional area of 2.56 cm^2 exposed to the receptor compartment. Phosphate buffer (pH 6.5) was used as a receptor phase and the temperature was maintained at 37°C. Vesicular and nonvesicular formulations (amount equivalent to 3.5 mg) were applied uniformly on the dorsal side of the skin. Aliquots of 5 ml were withdrawn periodically and replaced with fresh phosphate buffer (pH 6.5) to mimic infinite sink condition. The 5-FU content in samples was analyzed at 266 nm [18] using a UV-visible spectrophotometer.

Skin retention study

After completion of the skin permeation study, the skin mounted on the receptor chamber was removed and used to determine the amount of drug retained in the skin. The skin was properly cleaned by using cotton wetted in normal saline solution and placed on tissue paper to remove any formulation attached to the skin. The skin was then homogenized with 10 ml of chloroform:methanol mixture (2 : 1 v/v) to remove the drug retained in the skin. The suspension thus obtained was passed through a 0.22-μm membrane filter (MDI) and then quantified for the amount of drug retained at 266 nm [18] using a UV-visible spectrophotometer.

Cell cytotoxicity

HaCaT (nonmelanoma) cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamate, D-glucose, pyruvate, 10% fetal bovine serum (v/v), penicillin (100 UI/ml), and streptomycin (100 μg/ml). The medium was replaced with fresh DMEM medium every 48 h. HaCaT cells, presenting mutations in the p53 tumor suppressor gene, were cultured at 40°C for 1 week to obtain an in-vitro cell line model of nonmelanoma skin cancer. When 80% confluence was reached, cells were digested with trypsin and collected into a centrifuge tube containing 4 ml of the DMEM medium. The dishes were further washed with 2 ml of phosphate buffer solution to remove the remaining cells and this was transferred into a centrifuge

tube. Subsequently, the tube was centrifuged at 1000 rpm at room temperature for 10 min. The pellet was resuspended in an appropriate volume of culture medium and seeded in culture dishes. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a 96-well microtiter plate. Five thousand cells per well were plated onto 200 µl of the complete medium and exposed for 72 h with varying concentrations (0.01–20 µmol/l of 5-FU) of 5-FUAQ, 5-FUTR3, 5-FULS3, and 5-FUNS3. MTT assays were performed at the end of the treatment. Further control and treated cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37°C. The cells were lysed and the medium was removed. The formazan crystals were dissolved using 100 µl of dimethyl sulfoxide. The absorbance was recorded at 570 nm using 630 nm as a reference wavelength using an enzyme-linked immunosorbent assay reader (Tecan, Mannedorf, Switzerland).

Statistical analysis

The results are expressed as the mean ± standard deviation for *n* value of more than or equal to 3. Statistical significance was determined with a one-way analysis of variance test. A *P* value of less than 0.05 was taken as a significant level of difference.

Results and discussion

Transfersomes: preparation and characterization

Transfersomes contain lipid and a membrane-fluidizing agent, SDC. An optimum ratio of SPC and SDC could lead to a better deformability and entrapment efficiency. The entrapment efficiency of drug depends on the interaction between the drug and lipid bilayers, lipid concentration, and the number of extrusions as well. Different ratios (% w/w) of SPC and SDC were taken for the preparation of transfersomes (Table 1). It was observed that 5-FUTR3 (85 : 15% w/w SPC and SDC) produces transfersomes of 153.2 ± 10.3 nm in diameter with a maximum drug-entrapment efficiency of 82.4 ± 4.8%. Besides, 85 : 15% w/w of SPC and SDC produced size-controlled vesicles using the hydration method. TEM and AFM were used to characterize transfersomes. Transfersomes invariably appeared as unilammelar vesicles (Fig. 2a). In addition, 5-FUTR3 was found to have the most deformable vesicles, as the deformability index was found to be 130.4 ± 4.2. Zeta potential measurements showed better stability of transfersomes (– 39.6 ± 0.2) compared with liposomes (– 36.9 ± 0.1) and niosomes (– 33.4 ± 0.4; Table 2).

Liposomes and niosomes: preparation and characterization

Liposomes and niosomes were prepared using the reverse-phase evaporation technique, associated with relatively higher encapsulation efficiency of drugs [21]. The nature of the buffer used to dissolve the drug with respect to composition, pH, and molarity did not

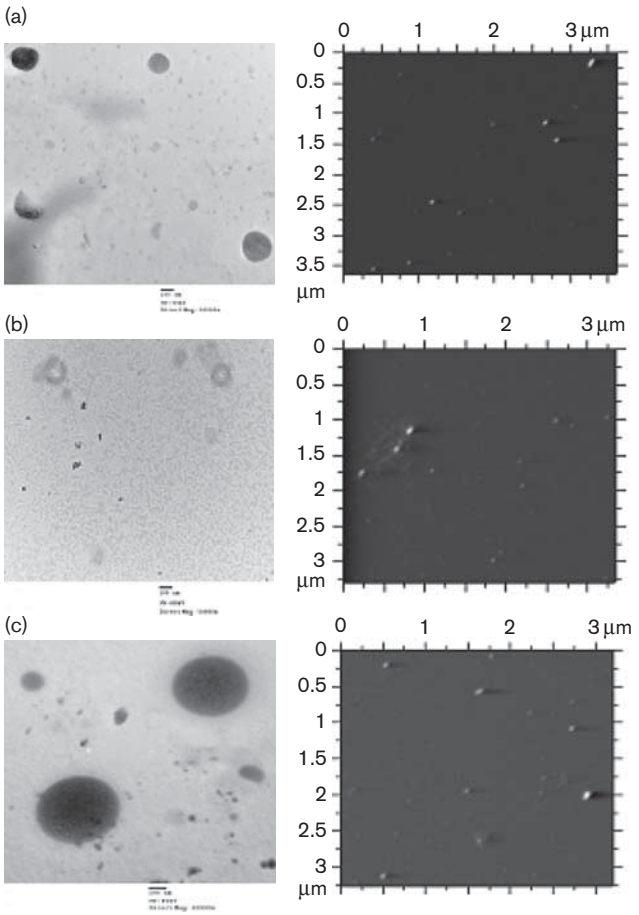
Table 1 Composition, particle size, and encapsulation efficiency of various formulations of 5-fluorouracil-loaded transfersomes, liposomes, and niosomes

	Transfersomes			Liposomes				Niosomes			
Formulation code	Composition (SPC: SDC; %w/w)	Size (nm)	Encapsulation efficiency (%)	Formulation code	Composition (SPC: cholesterol; mol/mol)	Size (nm)	Encapsulation efficiency (%)	Formulation code	Composition (SP 80: cholesterol; mol/mol)	Size (nm)	Encapsulation efficiency (%)
5-FUTR1	95 : 5	140 ± 4.5	78.1 ± 2.7	5-FULS1	9 : 1	105.6 ± 9.3	30.6 ± 3.2	5-FUNS1	9 : 2	235.3 ± 11.5	29.5 ± 2.3
5-FUTR2	90 : 10	148 ± 7.6	79.3 ± 3.1	5-FULS2	8 : 2	110.5 ± 15.4	38.9 ± 4.7	5-FUNS2	8 : 2	240.5 ± 9.8	37.3 ± 3.2
5-FUTR3	85 : 15	153.2 ± 10.3	82.4 ± 4.8	5-FULS3	7 : 3	120.3 ± 9.8	45.4 ± 3.3	5-FUNS3	7 : 3	250.4 ± 8.6	43.4 ± 3.2
5-FUTR4	80 : 20	200 ± 9.5	78.9 ± 3.4	5-FULS4	6 : 4	122.4 ± 5.6	37.3 ± 2.4	5-FUNS4	6 : 4	256.6 ± 15.4	35.3 ± 4.3
5-FUTR5	75 : 25	210 ± 14.3	76.4 ± 4.5	5-FULS5	5 : 5	125.5 ± 6.7	35.9 ± 3.8	5-FUNS5	5 : 5	260.2 ± 9.2	34.4 ± 3.2

5-FU, 5-fluorouracil; 5-FULS, 5-FU-loaded liposomes; 5-FUNS, 5-FU-loaded niosomes; 5-FUTR, 5-FU-loaded transfersomes; SDC, sodium deoxycholate; SPC, soya phosphatidylcholine.
Values are shown as mean ± standard deviation for $n \geq 3$.

interfere with either the formation or the yield of entrapment. The prepared vesicular systems were optimized with respect to particle size and entrapment efficiency. Different molar ratios of SPC and cholesterol were taken for the preparation of liposomes and niosomes (Table 1). The composition of 5-FULS3 (7 : 3 mol/l, SPC and cholesterol) produces liposomes of 120.3 ± 9.8 nm in diameter with an entrapment efficiency of 45.4 ± 3.3%. Moreover, 7:3 mol/l of SPC and cholesterol produced size-controlled vesicles using the hydration method followed by sonication [22]. Similarly, 5-FUNS3 (7 : 3 mol/l, SP 80 and cholesterol) produces niosomes of 250.4 ± 8.6 nm in diameter with a maximum entrapment efficiency of 43.4 ± 3.2%. Transfersomes showed a significantly higher entrapment efficiency in comparison with liposomes and niosomes, which may be attributed to the higher lipid content of transfersomes resulting in a higher entrapment efficiency [19] Moreover, transfersomes contain a mixture of lipids and membrane softeners, i.e. SDC. The lipid is a stabilizing factor and SDC is a destabilizing factor. During the preparation of transfersomes, the nanovesicle content is exchanged with the dispersion medium during breaking and resealing of the phospholipid bilayer as they pass through the polycarbonate membrane filters. Thus, a comparatively higher lipid concentration and successive extrusion through the membrane filters may synergistically lead to a higher entrapment efficiency of transfersomes in comparison with liposomes and niosomes The entrapment efficiency of liposomes and niosomes was almost equal; however, a slightly lower entrapment efficiency in the case of niosomes estimated may account for the pore-formation characteristics of the Span 80 in the bilayer of the niosomes [11]. TEM and AFM were used to characterize the shape and surface morphology of liposomes and niosomes (Fig. 2b and c). These nanovesicular carriers also invariably appeared as unilammelar vesicles. Moreover, 5-FULS3 and 5-FUNS3 have shown a deformability index of 31.25 ± 2.36 and 32.37 ± 3.2, respectively. It is well documented that lipid vesicle penetration through the skin is a function of carrier membrane deformability. Substances with particle size less than 500 Da can easily pass through the epidermis layer, but the larger particles must have considerable deformability to pass through the epidermis layer. Nanosized particles must be deformable enough so as to squeeze themselves between the pores of skin to find access to the target cell [23]. Prepared formulations were subjected to a deformability study by extrusion measurement. Deformability index was found to be maximal with the transfersomal formulation in comparison with liposomes and niosomes. This may be attributed to the fact that SDC mainly contribute to the transfersomal deformability, whereas cholesterol in liposomes and niosomes tends to form a rigid vesicle. Consequently, they are less capable of passing through the barrier with pores smaller than their own diameter. Liposomes and niosomes have almost a similar deformability index, but still niosomes have an edge over liposomes, the

Fig. 2



Transmission electron micrograph and atomic force microscopy of (a) transfersomes, (b) liposomes, (c) niosomes. The scale bar represents a distance of 100 nm.

Table 2 Zeta potential of optimized formulations of 5-fluorouracil-loaded and blank transfersomes, liposomes, and niosomes

Transfersomes		Liposomes		Niosomes	
Formulation code	Zeta potential (− mV)	Formulation code	Zeta potential (− mV)	Formulation code	Zeta potential (− mV)
5-FUTR3	− 39.6 ± 0.3	5-FULS3	− 36.9 ± 0.4	5-FUNS3	− 33.4 ± 0.2
BTR3	− 26.9 ± 0.2	BLS3	− 24.8 ± 0.1	BNS3	− 22.9 ± 0.3

BLS, BNS, and BTR, blank formulations of liposomes, niosomes, and transfersomes; 5-FU, 5-fluorouracil; 5-FULS, 5-FU-loaded liposomes; 5-FUNS, 5-FU-loaded niosomes; 5-FUTR, 5-FU-loaded transfersomes.
Values are shown as mean ± standard deviation for $n \geq 3$.

Table 3 Storage-stability profile of optimized formulation of 5-fluorouracil conducted for 5 weeks

	Temperature (°C)					
	4–8°C		25 ± 2°C		37 ± 2°C	
Formulation code	Particle size (nm)	Encapsulation efficiency (%)	Particle size (nm)	Encapsulation efficiency (%)	Particle size (nm)	Encapsulation efficiency (%)
5-FUTR3	155.5 ± 7.6	81.2 ± 4.1	159 ± 6.5	79.2 ± 4.5	184.5 ± 4.8	70.4 ± 5.2
5-FULS3	122.5 ± 5.7	45.1 ± 5.1	127 ± 3.5	41.2 ± 3.3	146.2 ± 4.2	36.7 ± 3.9
5-FUNS3	251.8 ± 10.6	42.1 ± 5.1	259 ± 2.5	36.4 ± 4.3	269.2 ± 3.3	31.2 ± 4.5

5-FULS, 5-FU-loaded liposomes; 5-FUNS, 5-FU-loaded niosomes; 5-FUTR, 5-FU-loaded transfersomes.
Values are shown as mean ± standard deviation for $n \geq 3$.

reason could be the membrane-softening effect of Span 80, which is a constitutive component of niosomes. Zeta potential values showed the increase in negative charge of 5-FULS3 (−36.9 ± 0.1) and 5-FUNS3 (−33.4 ± 0.4) when compared with the respective blank formulations (Table 2). The presence of a more negative charge on the surface of transfersomes further advocates the better stability of the same over liposomes and niosomes. Hence, the optimized batches (5-FUTR3, 5-FULS3, and 5-FUNS3) were then considered for in-vitro release and in-vitro cytotoxicity analysis.

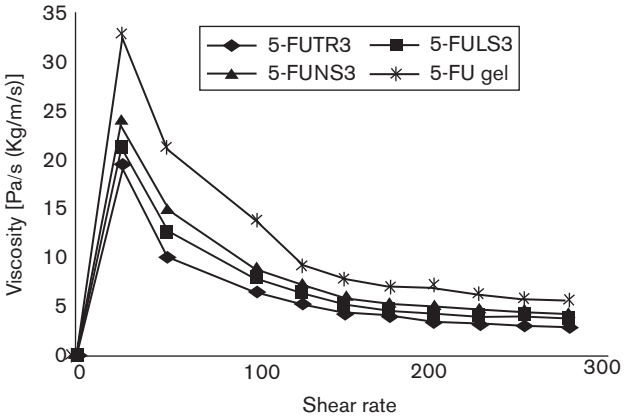
Stability profile

5-FUTR3 was found to entrap maximum 5-FU (70.4 ± 5.2%) even at elevated temperatures (37 ± 2°C). A substantial decrease in the entrapment efficiency of 5-FU was observed in 5-FULS3 (36.7 ± 3.9) and 5-FUNS3 (31.2 ± 4.5) at 37 ± 2°C. In contrast, at lower temperature conditions, that is, 4–8°C optimized batches (5-FUTR3, 5-FULS3, and 5-FUNS3) could retain 81.2 ± 4.1, 45.1 ± 5.1, and 42.1 ± 5.1% of 5-FU, respectively (Table 3). Acceleration in drug leakage at higher temperatures, as observed in storage-stability studies, suggested keeping the nanovesicles in refrigerated conditions, to minimize drug leakage from vesicular systems. Loss of drug from the nanovesicles stored at elevated temperatures may be attributed to the effect of temperature on the gel-to-liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in the membrane packing. The drug leakage of less than 5% of the initial load under refrigeration conditions is well within the limits, when vesicles are to be advocated for topical application.

Rheological analysis

Data indicate (Fig. 3) that there was no significant ($P > 0.05$) difference in viscosity of 5-FUTR3, 5-FULS3, and 5-FUNS3 when incorporated into a gel base. However, a significant difference ($P < 0.05$) was observed between the viscosity of nanovesicular (5-FUTR3, 5-FULS3, and 5-FUNS3) formulations incorporated into the gel base and nonvesicular preparations (5-FU gel). It may be attributed to the flexibility of lipoidal membrane of the nanovesicles. The presence of SPC and surfactant decrease the viscosity of nanovesicular formulations.

Fig. 3



Viscosity of 5-fluorouracil (5-FU) formulations at different shear rates. The result indicated no significant ($P > 0.05$) difference between the viscosities of 5-FU-loaded nanovesicular formulations, but the change in viscosity was significant (one-way analysis of variance; $P < 0.05$) when compared with nonvesicular formulation. 5-FULS, 5-FU-loaded liposomes; 5-FUTR, 5-FU-loaded transfersomes.

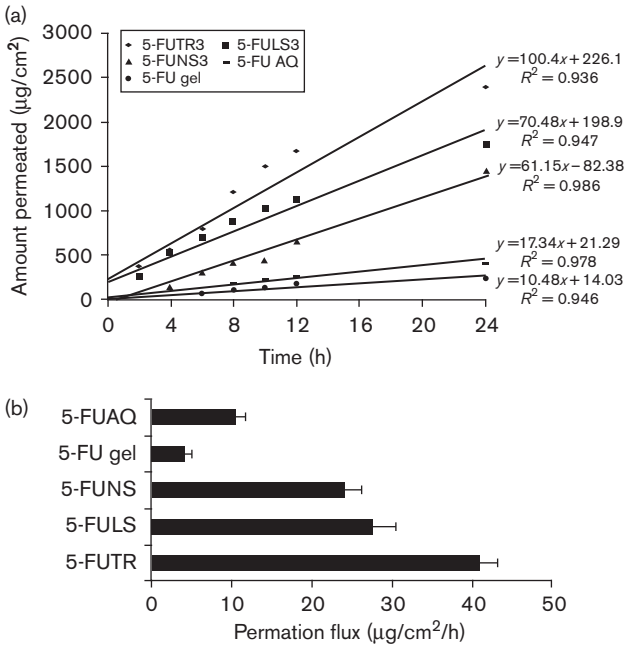
Slightly higher viscosity of 5-FUTR3 over 5-FULS3 and 5-FUNS3 may be attributed to the fluidity provided by SDC. As all the prepared nanovesicular formulations were found to follow the Newtonian law, they may be considered as a better future of topical drug delivery of 5-FU.

In-vitro skin permeation, release kinetic and retention study

A significant increase in skin permeation per unit surface area of 5-FU was observed (Fig. 4a) when optimized batches (5-FUTR3, 5-FULS3, and 5-FUNS3) were incorporated into the gel base. The amount of 5-FU permeated in 24 h was found to be 68.25 ± 5.36, 49.85 ± 4.65, and 41.14 ± 3.69% for 5-FUTR3, 5-FULS3, and 5-FUNS3, respectively; whereas only 7.0 ± 0.9 and 11.77 ± 1.21% of 5-FU was permeated from 5-FU gel and 5-FUAQ, respectively. First-order (log cumulative amount of drug released vs. time), Higuchi's equation (cumulative percent of drug released vs. square root of time), and zero order (cumulative percent of drug released vs. time) equations were applied to determine the release kinetics of 5-FU from 5-FUTR3, 5-FULS3, and 5-FUNS3-

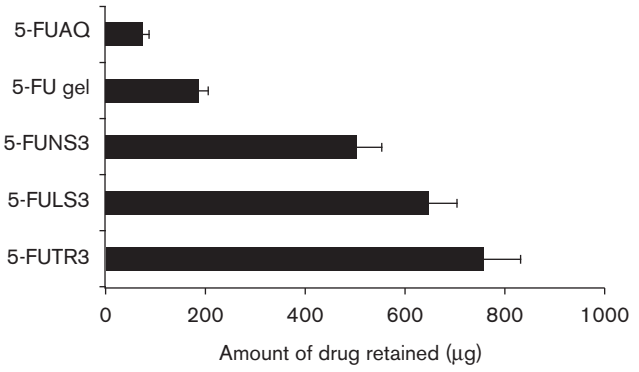
incorporated gel base system and compared with 5-FU gel and 5-FUAQ (Table 4). It was observed that all nanovesicle-incorporated gel formulations fit better into the first-order release kinetics rather than Higuchi-type and zero-order kinetics. First-order release kinetics is associated with burst release followed by slow release. Burst release and sustained release both are of interest for dermal application. Burst release can be useful to improve the penetration of drug, and sustained release supplied the drug over a prolonged period of time. Higher values of permeation flux (Fig. 4b) obtained with 5-FUTR3 ($40.8 \pm 1.69\,\mu g/cm^2/h$), 5-FULS3 ($27.53 \pm 1.56\,\mu g/cm^2/h$), and 5-FUNS3 ($23.88 \pm 1.31\,\mu g/cm^2/h$) than that of 5-FU gel ($4.09 \pm 0.93\,\mu g/cm^2/h$) and 5-FUAQ ($10.51 \pm 1.36\,\mu g/cm^2/h$) clearly advocates the permeation-enhancing effect of vesiculization of 5-FU. The bar diagram (Fig. 5) indicates the amount of 5-FU retained in the skin with its different formulation. The higher retention of drug by 5-FUTR3 ($756 \pm 69.58\,\mu g$), 5-FULS3 ($647 \pm 58.67\,\mu g$), and 5-FUNS3 ($504 \pm 48.52\,\mu g$) in comparison with 5-FU gel ($186 \pm 15.25\,\mu g$) may be concluded with the understanding that incorporation of 5-FU into transfersomes, liposomes, and niosomes not only enhance the penetration of the drug but also helped localize 5-FU in the skin. For optimum permeation, a drug must have lipid solubility high enough to facilitate the partitioning of the drug in the lipoidal biomembrane. The presence of lipid in the nanovesicles helped in generating and retaining the required physicochemical state of the skin for enhanced permeation and retention of 5-FU over the nonvesicular formulation. The phospholipid-rich domains of nanovesicles might have helped to produce the depot effect of drug molecules. The latter has been reflected in

Fig. 4



(a) Diagram depicting a graph of regressed line between 5-fluorouracil (5-FU) permeated and the time for optimized baths of 5-FU-loaded transfersomes (5-FUTR3), 5-FU-loaded liposomes (5-FULS3), 5-FU-loaded niosomes (5-FUNS3), 5-FU gel, and 5-FU-loaded aqueous solution (5-FUAQ). Results indicated that 5-FUTR3 shows maximum permeability followed by 5-FULS3 and 5-FUNS3. (b) Bar diagram depicting the flux of 5-FU permeated from the various formulations of 5-FU. The result indicated that maximum flux was obtained in 5-FUTR3 ($40.8 \pm 1.69\,\mu g/cm^2/h$) followed by 5-FULS3 ($27.53 \pm 1.56\,\mu g/cm^2/h$) and 5-FUNS3 ($23.88 \pm 1.31\,\mu g/cm^2/h$) than that of 5-FU gel (one-way analysis of variance; $P < 0.05$; $4.09 \pm 0.93\,\mu g/cm^2/h$) and 5-FUAQ ($10.51 \pm 1.36\,\mu g/cm^2/h$). Values are shown as mean ± standard deviation for $n \geq 3$.

Fig. 5



Bar diagram showing the amount of 5-fluorouracil (5-FU) retained in the rat skin with different formulations. Results indicated that maximum amount of 5-FU was retained with 5-FU-loaded transfersomes (5-FUTR3; 756 ± 69.58) followed by 5-FU-loaded liposomes (5-FULS3; 647 ± 58.67), and 5-FU-loaded niosomes (5-FUNS3; 504 ± 48.52) than that of 5-FU gel (one-way analysis of variance; $P < 0.05$; 186 ± 15.25). Values are shown as mean ± standard deviation for $n \geq 3$.

Table 4 Nonlinear fits of 5-fluorouracil released from different formulations

	Equation			R^2		
Sample	First order [ln (Q_0–Q) vs. t]	Higuchi (Q vs. $\sqrt{t}$)	Zero order (Q vs. t)	First order	Higuchi	Zero order
5-FUTR3	$y = -0.021x + 0.54$	$y = 0.520x - 0.264$	$y = 0.100x + 0.221$	0.988	0.947	0.936
5-FULS3	$y = -0.012x + 0.52$	$y = 0.368x - 0.153$	$y = 0.070x + 0.194$	0.987	0.974	0.949
5-FUNS3	$y = -0.009x + 0.562$	$y = 0.283x - 0.299$	$y = 0.060x - 0.084$	0.966	0.807	0.985
5-FU gel	$y = -0.002x + 0.542$	$y = 0.087x - 0.059$	$y = 0.017x + 0.017$	0.984	0.930	0.978
5-FUAQ	$y = 0.001x + 0.542$	$y = 0.052x - 0.037$	$y = 0.010x + 0.008$	0.948	0.897	0.949

5-FULS, 5-FU-loaded liposomes; 5-FUNS, 5-FU-loaded niosomes; 5-FUTR, 5-FU-loaded transfersomes; Q, amount of drug released at time t (mg); Q_0, drug to be released at zero time; t, time in hours.

the higher amount of drug retained within the skin layers in case of nanovesicular formulations. Furthermore, in-vitro skin permeation and retention was found to be best in the case of transfersomes in comparison with liposomes and niosomes; the reason behind this may be a higher lipid content and deformability. Thus, the vesicular 5-FU formulations, with desired characteristics for topical administration, could be successfully prepared. The formulated vesicular formulations have shown an appreciably enhanced skin permeation and retention of 5-FU molecules in the skin.

In-vitro cytotoxicity analysis

In-vitro cytotoxicity study on HaCaT (nonmelanoma) cancer cell lines describes better cytotoxicity of 5-FU on vesiculization. The IC_{50} value (Fig. 6) of 5-FUTR3 (1.02 μmol/l), 5-FULS3 (6.83 μmol/l), and 5-FUNS3 (9.91 μmol/l) was found to be far less than 5-FUAQ (15.89 μmol/l) at 72 h. 5-FUTR3 were found to be most cytotoxic on HaCaT cell line in comparison with 5-FULS3 and 5-FUNS3. The substantial decrease in IC_{50} values of transferosomes may be ascribed to high deformability, which lead to enhanced access to the inside of the cell. Liposomes and niosomes were also found to decrease the IC_{50} value of 5-FU. The reason behind the decrease in the IC_{50} values of 5-FU entrapped in transfersomes and liposomes may be imputed to an enhanced cellular uptake by virtue of which permeability of cell walls of tumor cell is increased for lipid formulations.

We have shown that docking of 5-FU in transfersomes, liposomes, and niosomes not only improves the topical release but also leads to a better cytotoxicity. The analysis of encapsulation efficiency showed that transfersomes can entrap more 5-FU than liposomes and niosomes. In addition, they are most deformable and can pass through pores of much less diameter than the transfersomes themselves. This shows that SDC provides maximum deformability to the vesicular membrane. Incorporation of these nanovesicles into the gel base leads to a better retention of 5-FU. Cytotoxicity analysis showed that vesiculization of 5-FU leads to a substantial decrease in the IC_{50} value of 5-FU. Encapsulation of 5-FU in transfersomes leads to considerable decrease in the IC_{50} value of 5-FU than liposomes and niosomes. We propose that transfersomes as a vesicular drug carrier not only overcome the demerits of traditional dosage form, but also the loopholes of liposomal and niosomal formulations are substantially removed. It creates a new opportunity for the well-controlled topical delivery of a number of drugs that have a problem of administration by other routes.

Fig. 6

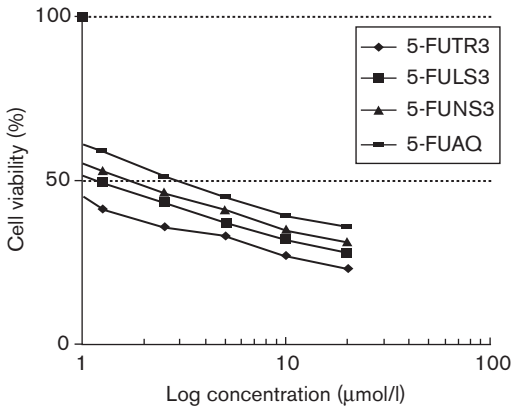


Diagram between the percent cell viability and 5-fluorouracil (5-FU) concentration (μmol/l) depicting the cytotoxicity of 5-FU formulations on HaCaT (nonmelanoma skin cancer cell line). The result indicates that IC_{50} values of 5-FU-loaded transfersomes (5-FUTR3; 1.02 μmol/l), 5-FU-loaded liposomes (5-FULS3; 6.83 μmol/l), and 5-FU-loaded niosomes (5-FUNS3; 9.91 μmol/l) were found to be far less (one-way analysis of variance; $P < 0.05$) than 5-FU-loaded aqueous solution (5-FUAQ; 15.89 μmol/l). Values are shown as mean ± standard deviation for $n \geq 3$.

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Conflicts of interest

There are no conflicts of interest.

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