Drug Development and Industrial Pharmacy, 2011; 37(12): 1491–1508

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI: 10.3109/03639045.2011.587431

informa
healthcare

RESEARCH ARTICLE

Niosomes as a potential drug delivery system for increasing the efficacy and safety of nystatin

Mohammed Shafik El-Ridy[1], Ahmed Abdelbary[2], Tamer Essam[3], Rania Mohsen Abd EL-Salam[4], and Ahmed Alaa Aly Kassem[1]

[1]Pharmaceutical Technology Department, Pharmaceutical and Drug Industries Research Division, National Research Center (NRC), Dokki, Cairo 12311, Egypt, [2]Pharmaceutics Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt, [3]Biotechnology Center (BTC), Microbiology and Immunology Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt, and [4]Pharmacology and Toxicology Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt

Abstract

Nonionic surfactant (NIS) vesicles (niosomes) formed from self-assembly of hydrated synthetic NIS monomers are capable of entrapping a variety of drugs and have been evaluated as an alternative to liposomes. Nystatin (NYS) is a polyene antifungal drug that has been used in the treatment of cutaneous, vaginal and oral fungal infections since the 1950s. The aim of this work is to encapsulate NYS in niosomes to obtain a safe and effective formula administered parenterally for neutropenic patients. NYS niosomes were prepared by the thin-film hydration method using Span 60 or Span 40 and cholesterol (CHOL). Stearylamine and dicetyl phosphate were added as the positive and negative charge-inducing agents (CIA), respectively. Two molar ratios were used, namely NIS/CHOL/CIA (1:1:0.1 and 2:1:0.25). Neutral and positively charged niosomes gave the highest encapsulation efficiencies. NYS niosomes were characterized using transmission electron microscopy, differential scanning calorimetry and dynamic light scattering. The release of neutral and negatively charged NYS niosomes was estimated, and it showed a slow sustained release profile. A 25-kGy γ-irradiation dose was sufficient to sterilize the investigated vesicles. NYS niosomes exerted less nephrotoxicity and hepatotoxicity *in vivo*, showed higher level of drug in vital organs and revealed pronounced efficacy in elimination of the fungal burden in experimental animals infected with *Candida albicans* compared with those treated with free NYS. Niosomal encapsulation thus provided means for parenteral administration of NYS, reducing its toxicity and making it a more active antifungal agent.

Keywords: Niosomes, Vesicular systems, Drug delivery, Drug targeting, Nystatin, Candidiasis, Immunosuppression

Introduction

The increasing burden of fungal infections, especially in markedly immunosuppressed patients and patients in the intensive care unit with multiple diseases, has created an urgent need for new antifungal agents[1]. Emergence of drug-resistant fungal pathogens led to the development of new antifungal agents and reengineering of currently available limited number of antifungal drugs[2]. Nystatin (NYS) is a polyene antifungal drug that has been used in the treatment of cutaneous, vaginal and oral fungal infections since the 1950s[3]. Topical and oral administration of NYS is not associated with significant toxicities, because the compound is not absorbed through the skin or from the gastrointestinal tract[2]. Previous attempts to administer NYS systemically in the clinical setting resulted in sclerosing of the veins accompanied by severe shaking, chills, fever and malaise[4]. This eventually resulted in the discontinuation of NYS therapy. To circumvent the problems associated with systemic administration of free NYS, a liposomal formulation of NYS (Nyotran®) was recently developed by Aronex Pharmaceuticals Inc[2]. Liposomal NYS has been found to be less toxic and more effective in the treatment of *Candida albicans* and *Aspergillus fumigatus* infections in mice compared with equivalent dose of free NYS[5,6]. However, there remain significant problems in the general applications of liposomes for drug delivery. Some major disadvantages

Address for Correspondence: Mohammed Shafik El-Ridy, Pharmaceutical Technology Department, National Research Center, El Bohous Street, Dokki, Cairo 12311, Egypt. Tel.: +202 33335456. Fax: +202 33370931. E-mail: dr_elridy@yahoo.com

(Received 08 March 2011; revised 29 April 2011; accepted 06 May 2011)

associated with liposomes include degradation by hydrolysis or oxidation, sedimentation, leaching of drugs and aggregation or fusion of liposomes during storage[7–9]. Niosomes exhibit a behavior similar to liposomes *in vivo*[10] and have distinct advantages over liposomes. They possess greater stability and avoid disadvantages associated with liposomes such as variable purity of phospholipids and requirement of inert conditions for processing. Relatively low cost of materials makes them suitable for industrial manufacture[11–13]. In addition, an increase in numbers of nonionic surfactants (NISs) gives a variety of choices for selection and leads to capability of entrapping both hydrophobic and hydrophilic solutes[14,15]. Because of the technological benefits, niosome as a novel carrier has found broad attention among the pharmaceutical researchers of late[16]. In this study, NYS niosomes were prepared, characterized, monitored for toxicity and evaluated against *C. albicans* disseminated mice to approach an efficient and safe formulation that overcome disadvantages associated with liposomes and toxicity with free NYS.

Materials and methods

Chemicals

NYS was a gift sample from the Tenth of Ramadan Company for Pharmaceutical Industries and Diagnostic Reagents (RAMEDA), Cairo, Egypt, originally obtained from Bouchara Recordati Laboratories, France. Sorbitan monostearate (Span 60) (Merck Chemie, Germany), sorbitan monopalmitate (Span 40), cholesterol (CHOL) from lanolin, minimum 99%, dicetyl phosphate (DCP) and stearylamine (SA) minimum 99% were purchased from Sigma-Aldrich Chemie, Germany, dimethyl sulfoxide (DMSO), high-performance liquid chromatography (HPLC) grade, (Labscan Ltd., Ireland). All other chemicals were of analytical grade.

Animals

Adult female albino Wistar rats, weighing 150–200 g, and adult male albino mice weighting 20 ± 2 g, strain CD_1, were purchased from the animal house of the National Research Center, Cairo, Egypt. Animals were housed under constant environmental conditions and were allowed free access to water and food throughout the period of investigation. Animals were checked daily for their mortality and morbidity. The techniques used for bleeding, injecting and sacrificing of the animals were strictly performed following the mandates approved by the Animal Ethics Committee (Medical Research Ethics Committee (MREC), National Research Center, Cairo, Egypt).

Methods

Preparation of niosomes

The composition of the prepared niosomal formulations is shown in Table 1. Niosomes were prepared by the thin-film hydration method originally discussed by Bangham et al.[17] for liposomes and by Baillie et al.[18] for niosomes preparation. In brief, accurately weighed 5 mg of NYS was solubilized in 5 ml methanol (1 mg/ml) and sonicated in a bath-type sonicator for 5 min till obtaining a clear solution. In a 100-ml pear-shaped flask of the (Büchi-M/HB-140, Switzerland) rotary evaporator, 100 mg of NIS and CHOL with or without charge-inducing agent (CIA) were completely dissolved in 5 ml chloroform. The NYS solution was added to the flask getting a mixture of chloroform/methanol (1:1, vol/vol).[2,5,19–27] All ingredients, along with NYS (drug/ingredients, 1:20 w/w)[2,24], were dissolved and carefully evaporated under reduced pressure, on a water bath at 60°C, to form a thin film on the wall of the flask. The thin film was hydrated with 10 ml normal saline[2,5,19–21,24–27] under rotation at 60°C. The nonhydrated film traces on the wall of the flask were sonicated in the bath-type sonicator to ensure complete hydration of the niosomal film.

Determination of NYS entrapment in vesicles

To determine the amount of drug entrapped in niosomes, the unentrapped NYS was separated from the niosome-entrapped NYS by cooling centrifugation (5200*g*) at 4°C using the refrigerated centrifuge (Union 32R, Hanil

Table 1. Composition of the prepared niosomal formulations.

		Molar Ratio				
		Nonionic Surfactant			Charge-Inducing Agent	
Formula	Niosomes Type	Span 60	Span 40	CHOL	DCP	SA
F_1	Neutral niosomes	1	—	1	—	—
F_2	Negatively charged niosomes	1	—	1	0.1	—
F_3	Positively charged niosomes	1	—	1	—	0.1
F_4	Neutral niosomes	2	—	1	—	—
F_5	Negatively charged niosomes	2	—	1	0.25	—
F_6	Positively charged niosomes	2	—	1	—	0.25
F_7	Neutral niosomes	—	1	1	—	—
F_8	Negatively charged niosomes	—	1	1	0.1	—
F_9	Positively charged niosomes	—	1	1	—	0.1
F_{10}	Neutral niosomes	—	2	1	—	—
F_{11}	Negatively charged niosomes	—	2	1	0.25	—
F_{12}	Positively charged niosomes	—	2	1	—	0.25

Science Industrial Co., Ltd., Korea) for 30 min[28–30]. The niosomal pellets were then washed once with 30 ml normal saline. The pellets were resuspended in 10 ml normal saline[27], and the amount of NYS incorporated in niosomes was determined by lysis of 1 ml of the resuspended pellets using methanol[2,5,19–24,27] followed by sonication in the bath-type sonicator for 30 min. A clear solution is obtained, which is measured spectrophotometrically at 304 nm[31] against drug-free niosomes, treated by the same way, as a blank. The percentage of drug entrapment was calculated.

Entrapment efficiency (EE%) is expressed as (amount of drug entrapped/total amount of drug added) × 100.

Characterization of niosomes

Transmission electron microscopy. The morphology of NYS niosomes and drug-free niosomes was characterized by transmission electron microscopy (TEM). One drop of the sample was placed onto a 300-mesh formvar carbon-coated grid and allowed to dry to a thin film. Before complete drying of this film on the grid, it was negatively stained with 1% phosphotungstic acid. One drop of the freshly prepared stain was added and allowed to air dry on a filter paper. After drying, the samples were then examined by the transmission electron microscope (JEOL, JEM-1230, Tokyo, Japan.), with an accelerating voltage of 80 kV. Photographs were taken at suitable magnifications.

Differential scanning calorimetry. The thermal properties were analyzed using differential scanning calorimetry (DSC; Shimadzu, DSC-60 with TA-60 WS thermal analyzer, Tokyo, Japan) calibrated with indium. Thermograms were analyzed using Shimadzu TA-60 software. The dehydrated niosomal formulations were used for the investigation, in addition to pure Span 60, Span 40, CHOL, DCP, SA and NYS. A sample (2–4 mg) of powder was placed in a hermetically sealed aluminum pan and scanned at a rate of 5°C/min over a temperature range of 20–120°C with nitrogen purging (5 ml/min). Alumina powder was used as the reference material in the DSC runs. The peak transition temperature (T_c) and enthalpy of transition (ΔH) were determined for each peak.

Determination of vesicle size. Niosomal vesicle size was determined by dynamic light scattering based on laser diffraction in a multimodal mode using the Nicomp particle sizing system (Zeta Potential/Particle Sizer, NICOMP™ 380 ZLS, Santa Barbara, CA) with He–Ne laser at 632.8 nm[32] at a scattering angle of 90.0°, which is capable of measuring vesicles in the 1 nm to 5 μm size range. The niosomal preparation was diluted with bidistilled water (1:100 vol/vol).[33–35] After dilution, the sample was transferred to a quartz cuvette and measured at room temperature. Size distributions were displayed in term of number versus vesicle size. The polydispersity index (PI) was determined as a measure of homogeneity. Small values of PI (<0.1) indicate a homogenous population, whereas high PI values (>0.3) indicate heterogeneity[36,37].

In vitro release profile studies of NYS niosomes. The *in vitro* release profile study of NYS niosomes was performed as a trial to anticipate the expected behavior of NYS niosomes under the hydrodynamic stress and body temperature of *in vivo* conditions. The method adopted for this examination was previously discussed by El-Ridy et al. and Mokhtar et al.[28,30] This study was performed on neutral and negatively charged niosomal formulations, namely F_1, F_2, F_4, F_5, F_7, F_8, F_{10} and F_{11} NYS niosomes. It should be noted that positively charged NYS niosomes, F_3, F_6, F_9 and F_{12}, were omitted from this experiment because of reported toxicity[38] and aggregation[39] *in vivo*. Each niosomal formulation was separated and washed, and the amount of NYS entrapped was determined spectrophotometrically at 304 nm. The amount entrapped in each niosomal formulation was considered as the total amount of drug retained at zero time, i.e. 100%. The pellet of each preparation was then suspended using normal saline to exactly 10 ml. The (Hilab, GLF 3202, Germany) rotary shaker was adjusted to a rate of 150 strokes/min and a temperature of 37 ± 0.2°C. A 1-ml sample from each of the niosomal suspensions was taken at different time intervals, namely at 3, 6, 24, 48 and 72 h after the start of the experiment. The samples were separated and washed, and the amount of NYS retained was determined at each time interval by spectrophotometric assay at 304 nm. The mean amount of NYS released was then calculated at each time interval for each of the formulae investigated. The mechanism of NYS release from niosomal formulations was determined using the following mathematical models: zero-order kinetics (cumulative % drug released vs. time), first-order kinetics (log % drug retained vs. time), second-order kinetics (inverse % drug retained vs. time) and Higuchi model (cumulative % drug released vs. square root of time). The R^2 and K values were calculated for the linear curve obtained by regression analysis of the above plots.

Sterilization of NYS niosomes by γ-irradiation

The investigation was performed to obtain the suitable dose of irradiation to sterilize the vesicles to be administered parenterally. For this study, two niosomal formulations, F_1 and F_{10} NYS niosomes, of the highest EE% were chosen. Niosomal formulations were transferred to glass vials and exposed to γ-irradiation using a Cobalt-60 source at ambient temperature by Canadian Gamma Cell. Two gamma irradiation doses were attempted for irradiation of NYS niosomes, *viz.* 15 and 25 kGy, and a control group was left unirradiated.

Sterility testing. The biological indicator used, *Bacillus pumilus* E601, is recognized as one of the challenge microorganisms used for the certification of commercial radiation sterilization cycles[40]. Two test pieces of the biological indicator, *B. pumilus* E601, were irradiated with the vials at each of the two irradiation doses, whereas two test pieces were left as control. The medium used for sterility testing was Brewer's thioglycollate medium. The content of each

vial was taken using a sterile syringe and inoculated into a sterile test tube with the thioglycollate medium. One test piece of the biological indicator *B. pumilus* E601 was inoculated into a sterile test tube of thioglycollate medium at each dose of irradiation. Two other sterile thioglycollate tubes were inoculated with broth culture of *Escherichia coli* and *Clostridium sporogenes* as aerobic and anaerobic bacteria, respectively. These tubes were considered as positive control to test the growth-promoting ability of the medium for both aerobes and anaerobes. One sterile test tube of sterile thioglycollate slant was left uninoculated, which acts as negative control, i.e. to check the sterility of the medium. All tubes were incubated at 32°C for 14 days and were examined regularly to monitor any growth.

Effect of γ-irradiation on the stability of the formulation. The effect of the irradiation process on the stability of the formulations was monitored by determining the NYS niosomes' EE% before and after the irradiation procedure. One milliliter sample was taken, separated, washed and assayed spectrophotometrically at 304 nm using methanol.

In vivo *renal and hepatic toxicity*

Renal and hepatic toxicity was monitored by applying five daily consecutive doses regimen to determine biochemical profiles of blood urea nitrogen (BUN), serum creatinine, aspartate transaminase (AST/sGOT) and alanine transaminase (ALT/sGPT). Two niosomal formulations, F_1 and F_{10} NYS niosomes, were used to compare their biochemical profile with free NYS dissolved in DMSO/saline (1:1 vol/vol), in addition to F_1–drug-free niosomes. A group receiving DMSO/saline (1:1 vol/vol) was considered as a control.

Experimental design

Fifty female Wistar rats weighing 150–200 g were randomly allocated into five groups, each containing 10 animals. The first group received F_1–NYS niosomes (5 mg/kg).[2,19–21] The second group received F_{10}–NYS niosomes (5 mg/kg). The third group received free NYS (5 mg/kg) dissolved in DMSO/saline (1:1 vol/vol). The fourth group received F_1–drug-free niosomes. The fifth group received DMSO/saline (1:1 vol/vol) as control. Treatment was performed daily for 5 consecutive days. Animals were injected intraperitoneally by a volume of 2 ml equivalent to the therapeutic dose of NYS (5 mg/kg).

Biochemical tests

On day 6, blood samples were collected from the retro-orbital plexus of each animal using a capillary tube. The blood was allowed to clot at room temperature, and serum was separated for the estimation of biochemical profiles of serum transaminases, creatinine and BUN using the appropriate test reagent kit.

Histopathologic studies

On day 6, livers and kidneys were isolated from two rats of each group for histopathologic examinations. Autopsy samples were taken from the liver and kidney of rats in different groups and fixed in 10% formol saline for 24 h. Tissues were processed and sectioned. The obtained tissue sections were collected on glass slides, deparaffinized, stained with hematoxylin and eosin and examined through the light electric microscope[41].

Tissue distribution studies

This study is conducted to compare the level of NYS distributed in the lungs, livers and spleens of mice after injecting a single dose of drug niosomes and its free form. Two niosomal formulations, F_1 and F_{10} NYS niosomes, and free NYS dissolved in DMSO/saline (1:1 vol/vol) were injected to compare the tissue levels, in addition to a control group receiving DMSO/saline (1:1 vol/vol).

Experimental design

Forty mice were divided randomly into four groups, each consisting of 10 mice. The first group received F_1–NYS niosomes (5 mg/kg).[2,19–21] The second group received F_{10}–NYS niosomes (5 mg/kg).The third group received free NYS (5 mg/kg) dissolved in DMSO/saline (1:1 vol/vol). The fourth group received DMSO/saline (1:1 vol/vol) as control. All groups were injected intraperitoneally with a single dose by a volume of 0.2 ml equivalent to the therapeutic dose of NYS (5 mg/kg).

At 24 and 48 h after injection, three animals from each group were sacrificed. The lungs, livers and spleens were separated and stored at –20°C. Before assay, tissues were thawed and rinsed with phosphate-buffered saline, pH 7.4. Buffer solution remaining on the tissue surface was blotted with wipes. Tissues were weighed and homogenized with HPLC-grade methanol. Tissues were homogenized in a high-speed tissue homogenizer twice for 30 s each time. Standard samples of the tissues for the drug analysis were prepared in the same manner by homogenizing normal tissues in HPLC-grade methanol and adding known amount of bulk NYS. Homogenized samples were centrifuged at 5200*g* for 15 min. The supernatant was taken, and a 1-ml portion of the methanolic supernatant was filtered through a 0.22-μm pore size syringe filter and submitted to assay. Drug concentrations in tissues were calculated to 1 g of tissue[42,43].

Analytical method

Levels of drug tissue homogenates were determined as total unassociated (free) NYS using a fully validated HPLC method[43] and bulk NYS as the reference standard using (Waters full equipped HPLC system, USA). The mobile phase consisted of 10 mM sodium phosphate monobasic, 1 mM EDTA, 30% HPLC-grade methanol, and 30% HPLC-grade acetonitrile adjusted to pH 6.0 by 85% phosphoric acid. The injection volume was 20 μl, and the flow rate was 1.0 ml/min. NYS was detected by UV absorbance at 304 nm using a C_{18} analytical column maintained at 30°C (phenomenex C_{18}; 250 × 4.6 mm; internal diameter, 100 Å; particle size, 5 μm). Quantification was based on the peak area concentration of NYS, which

eluted at 7.5–8.5 min, and the peak area concentration response of the external calibration standard[42–44].

Antifungal efficacy

Antifungal efficacy of NYS entrapped in niosomes and free NYS was evaluated by testing the reduction of the fungal load, i.e. colony-forming unit (CFU) on immunocompromised mice infected with *C. albicans* compared with nontreated mice acting as control, when administered parenterally.

Induction of immunosuppression

A single intraperitoneal injection of cyclophosphamide (250 mg/kg)[19,20,45] was given to each mouse to induce neutropenia that persists temporarily for 5–7 days after cyclophosphamide treatment[45,46]. Cyclophosphamide (Endoxan®) vials were reconstituted with water for injection, and each mouse was injected with 0.2 ml of cyclophosphamide solution (5 mg/0.2 ml/mouse). The animals were subsequently exposed to *C. albicans* infection on day 4 post-cyclophosphamide treatment, expecting the infection to occur under neutropenic conditions.

Inoculum and animal infection

C. albicans was grown on Sabouraud dextrose (SD) agar plates, supplemented with ampicillin and gentamicin. Few isolated colonies were spiked and dispersed into test tubes containing 10 ml normal saline. Then, the inoculum size was adjusted to 3.5×10^6 CFU/ml before animal infection. On day 4 postimmunosuppression, animals were intravenously injected (lateral tail vein) with 7×10^5 viable CFU of *C. albicans*, suspended in 0.2 ml of normal saline solution[5,19–21]. Infected animals were incubated for 24 h before the treatment regimen.

Experimental design

Twenty-four hours postinfection, 50 mice were randomly allocated into five groups, each containing 10 animals. The first group received F_1–NYS niosomes (5 mg/kg[2,19–21]). The second group received F_{10}–NYS niosomes (5 mg/kg). The third group received free NYS (5 mg/kg) dissolved in DMSO/saline (1:1 vol/vol). The fourth group received F_1–drug-free niosomes. The fifth group received DMSO/saline (1:1 vol/vol) as control. Treatment was performed daily for 5 consecutive days. Animal were injected intraperitoneally by a volume of 0.2 ml equivalent to the therapeutic dose of NYS (5 mg/kg).

Quantitative assessment of fungal load in various vital organs

The effect of treatment with niosomal NYS on the elimination of *C. albicans* infection was assessed by determining the fungal burden in various vital organs of the treated mice on day 6 postinfection. Three animals from each group were sacrificed and organs, *viz*. liver, lungs, spleen, kidney and heart were analyzed for their fungal load. Mice were dissected, and vital organs were isolated aseptically, washed with phosphate-buffered saline (pH 7.4) and kept frozen at –20°C. Each organ was weighed and homogenized in a tissue grinder for 15–30 s using 5 ml of sterile normal saline solution. Series of 10-fold dilutions were prepared for each organ. One milliliter of each dilution was cultured onto SD agar plates containing ampicillin and gentamicin. The plates were incubated at 37°C for 48 h. The number of colonies was counted, and the fungal load in various vital organs was determined by multiplying with the dilution factor[2,19–21]. Each set of experiment was conducted in triplicates. Percentage reduction of the fungal load was expressed as: % reduction = (mean CFU/g of control group – mean CFU/g of treatment group) × 100/(mean CFU/g of control group).

Data analysis and statistics

Results are expressed as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance followed by least significant difference multicomparisons test using SPSS (version 15.0). Significance was defined at P values <0.05.

Results and discussion

Entrapment efficiency

NYS entrapment was influenced by the affinity of the drug for the niosomes' materials, the thickness of the niosomes bilayers and the compatibility between the drug and niosomes' materials[47]. The EE% of all niosomal formulations are shown in Table 2. NYS was successfully entrapped in all the formulations prepared. The effect of several factors, including the effect of molar ratio (CHOL content), presence and absence of different surface charges and surfactant type, was evaluated.

Effect of molar ratio (CHOL content)

Many NISs form vesicles when CHOL is included in the bilayer to the level of 30–50 mole%[48]. In this study, both molar ratios investigated, namely NIS/CHOL/CIA (1:1:0.1 and 2:1:0.25) had 30–50 mole% CHOL, where all 12 formulae, F_1–F_{12}, could obviously trap NYS efficiently (Table 2). The results reveal that negatively and positively charged NYS niosomes, F_2, F_3, F_8 and F_9, of the molar ratio NIS/CHOL/CIA (1:1:0.1) possessed higher entrapment efficiencies ($P < 0.05$) in contrast to F_5, F_6, F_{11} and F_{12} NYS niosomes, at the molar ratio NIS/CHOL/CIA (2:1:0.25) of same surface charges. Same results were observed for neutral NYS niosomes ($P < 0.05$), F_1, of the molar ratio Span 60/CHOL (1:1) compared with F_4 of the molar ratio Span 60/CHOL (2:1). Neutral NYS niosomes, F_7 and F_{10}, of the molar ratios Span 40/CHOL (1:1 and 2:1) showed nearly similar encapsulation percentages with statistical nonsignificant difference ($P > 0.05$), *viz.* 88.26 and 89.64%, respectively. These findings might be attributed to the higher CHOL content, *viz.* ≈50 mole% for the molar ratio NIS/CHOL/CIA (1:1:0.1) compared with the molar ratio NIS/CHOL/CIA (2:1:0.25) with a CHOL content

Table 2. Entrapment efficiency (EE%), vesicle size and $Q_{72\,h}$ of the prepared nystatin niosomes.

Niosomal Formulation	(EE%)* ± SD	Vesicle Size (nm ± SD)	Variance (PI)†	$Q_{72\,h}$‡ (% ±SD)
F_1	94.34 ± 3.67	395.8 ± 53.4	0.135	8.82 ± 0.21
F_2	74.81 ± 2.10	392.9 ± 40.0	0.102	14.42 ± 0.52
F_3	97.88 ± 1.58	290.3 ± 39.7	0.137	—
F_4	85.96 ± 2.09	230.7 ± 28.1	0.122	8.23 ± 0.07
F_5	39.97 ± 3.57	270.6 ± 38.4	0.142	22.79 ± 0.55
F_6	91.98 ± 2.24	245.6 ± 32.6	0.133	—
F_7	88.26 ± 0.85	309.6 ± 29.7	0.096	13.97 ± 0.25
F_8	60.32 ± 2.12	299.3 ± 37	0.124	18.75 ± 2.50
F_9	89.31 ± 3.01	267.8 ± 30.4	0.114	—
F_{10}	89.64 ± 2.18	164.8 ± 22.3	0.135	13.46 ± 0.20
F_{11}	16.20 ± 2.87	213.7 ± 21.7	0.102	32.65 ± 4.82
F_{12}	73.48 ± 2.21	201.8 ± 25.6	0.127	—

*Each value is a mean of six to eight determinations.
†PI: polydispersity index obtained as PI = (SD/mean vesicle size).
‡$Q_{72\,h}$: percent nystatin released after 72 h.

of ≈30 mole%. CHOL is one of the common additives included in the formulation to prepare stable niosomes. CHOL stabilizes bilayers, prevents leakiness and retards permeation of solutes enclosed in the aqueous core of these vesicles[49]. Inclusion of CHOL increases the viscosity of the formulation, indicating more rigidity of the bilayer membrane. Moreover, drug partitioning will occur more easily in highly ordered systems of surfactant and CHOL[50]. This increase may be due to an increase in the lipophilic behavior of the surfactant bilayer of niosomes and crystallinity of the bilayer[11].

Effect of surface charge

Charged molecules, DCP and SA as a negative and positive charge inducers, respectively, are often used to increase stability of niosomal dispersions[49,51] and maintain their integrity and uniformity[50,52]. DCP is used to prevent niosome aggregation[49]. The results shown in Table 2 show that neutral and positively charged NYS niosomes, F_1, F_3, F_4, F_6, F_7, F_9, F_{10} and F_{12}, had significantly ($P < 0.05$) higher entrapment efficiencies compared with negatively charged NYS vesicles, F_2, F_5, F_8 and F_{11}. The results also depict that positively charged NYS niosomes, F_3 and F_9, revealed a nonsignificant ($P > 0.05$) higher encapsulation percentage compared with neutral NYS niosomes, F_1 and F_7. As the same time as, positively charged NYS niosomes, F_6, revealed a significant ($P < 0.05$) higher encapsulation efficiency compared with neutral NYS niosomes, F_4. On the contrary, neutral NYS niosomes, F_{10}, of the molar ratio Span 40/CHOL (2:1) revealed a significant ($P < 0.05$) higher EE% compared with positively charged NYS niosomes, F_{12}. Markedly, significant ($P < 0.05$) low EE% values for negatively charged NYS niosomes, F_2, F_5, F_8 and F_{11}, was observed. This could be possibly explained by the presence of a carboxyl group in the NYS molecule[53]. Under the experimental conditions, the carboxyl group would lose one proton and transform itself into a carboxylate anion. Obviously, the lower drug entrapment in the presence of DCP was due to the electrostatic repulsion forces between the carboxylate anion of NYS and the anionic head group of DCP[30,54,55]. On the other hand, imparting a positive charge to the niosomal vesicles resulted in a relatively higher NYS encapsulation percentage. This could be due to the electrostatic attraction between the positively charged head group in SA and the carboxylate anion in the dissociated NYS molecule[30,56].

Effect of NIS structure

An increasing number of NISs have been found to formulate niosomes, among which sorbitan esters are much more common[57]. It was reported that the length of alkyl chain is a crucial factor of permeability and that long-chain surfactants produce high drug entrapment into vesicles[58]. Span 60 and Span 40 are solid at room temperature with the highest transition temperatures. Therefore, in this study, Span 60 (C_{18}) and Span 40 (C_{16}) were chosen as the surfactants of choice for the formation of the nonionic vesicles. The results presented in Table 2 show that negatively and positively charged NYS niosomes, F_2, F_3, F_5 and F_6, of the molar ratios Span 60/CHOL/CIA (1:1:0.1 and 2:1:0.25) acquired significantly higher entrapment efficiencies ($P < 0.05$) in contrast to F_8, F_9, F_{11} and F_{12} of the molar ratios Span 40/CHOL/CIA (1:1:0.1 and 2:1:0.25) of same surface charges. Same results were observed ($P < 0.05$) for neutral NYS niosomes, F_1, of the molar ratio Span 60/CHOL (1:1) compared with F_7 of the molar ratio Span 40/CHOL (1:1). On the contrary, neutral NYS niosomes, F_{10}, of the molar ratio Span 40/CHOL (2:1) possessed statistically nonsignificant ($P > 0.05$) higher encapsulation percentage in contrast to F_4 NYS niosomes of the molar ratio Span 60/CHOL (2:1). The higher entrapment possessed by niosomes prepared using Span 60 may be due to the solid nature, hydrophobicity and high phase transition temperature of the surfactant, *viz.* Span 60[59]. It was reported that the higher the transition temperature of the surfactant the higher the encapsulation efficiency[11].

These findings are in agreement with previous works, where the increase in the alkyl chain length, Span 60 (C_{18}) > Span 40 (C_{16}), has led to an increase in the EE%[16,30,49,54,58,60,61]. In addition, the alkyl chain length influences the hydrophilic-lipophilic balance (HLB) value of the surfactant, which in turn directly influences the drug EE%[62,63]. The lower the HLB of the surfactant the higher will be the drug EE% and stability[57,64,65] as in the case of niosomes prepared using Span 60, HLB = 4.7, compared with Span 40 with a higher HLB of 6.7.

Characterization of niosomes

Transmission electron microscopy

Figure 1 shows the different vesicle shapes and sizes, at different magnification powers, of NYS niosomes and drug-free niosomes. All fields were negatively stained with 1% phosphotungstic acid at room temperature. It is demonstrated that the vesicles are well identified and present in a nearly perfect sphere-like shape having a large internal aqueous space and a smooth vesicle surface. Figure 1 reveals well-stained niosomal vesicles, where the outer lipophilic domain is black stained and the inner hydrophilic domain is light stained.

Differential scanning calorimetry

Figures 2 and 3 reveal the phase transition temperatures (T_c) of vesicles composed of Span 60 or Span 40/CHOL with or without CIA. The (T_c) was determined from the midpoint (main phase transition) temperature of the endothermic peaks[66,67]. The (T_c) for Span 60, Span 40 and NYS was 54.20, 51.13 and 168.86°C, respectively (Figure 2), representing their gel–liquid transition temperature. Considering Span 60 and Span 40 niosomes, an obvious decrease in T_c was noticed with clear changes in the enthalpy in addition to a considerable peak broadening. Formation of niosomes leads to a change in transition temperature of Span 60 and Span 40 accompanied by a change in the heat of fusion regarding most of the charges investigated. All the thermodynamic changes for NYS niosomes, drug-free niosomes and their individual components investigated are shown in Figures 2 and 3. This agrees with the fact that at room temperature (25°C), molecules of Span 60 and Span 40 in bilayer structures are in the ordered gel state[59]. The NYS-loaded niosomes showed disappearance of the melting endotherm of NYS and broadening of the NIS's endothermic peak. This may suggest that NYS can fit into the niosomal bilayer producing significant perturbation in the packing characteristics of the niosomal membrane and accordingly was able to reduce the peak of the main transition of the NIS[57,68,69].

Determination of vesicle size

Vesicle size is an important parameter that influences the biopharmaceutical feature of the carrier[34]. For this reason, the vesicle size was evaluated as a function of the formulation parameters. The vesicle sizes of F_1–F_{12} NYS niosomes are presented in Table 2.

Effect of CHOL content

The results (Table 2) show that the vesicle sizes of Span 60 and Span 40 NYS niosomes of the molar ratio NIS/CHOL/CIA (1:1:0.1), F_1–F_3 and F_7–F_9 were markedly larger than the molar ratio NIS/CHOL/CIA (2:1:0.25), F_4–F_6 and F_{10}–F_{12}. These results are in agreement with the previous studies showing that an increment in the amount of CHOL caused the size of vesicles to increase[47,67,70–72].

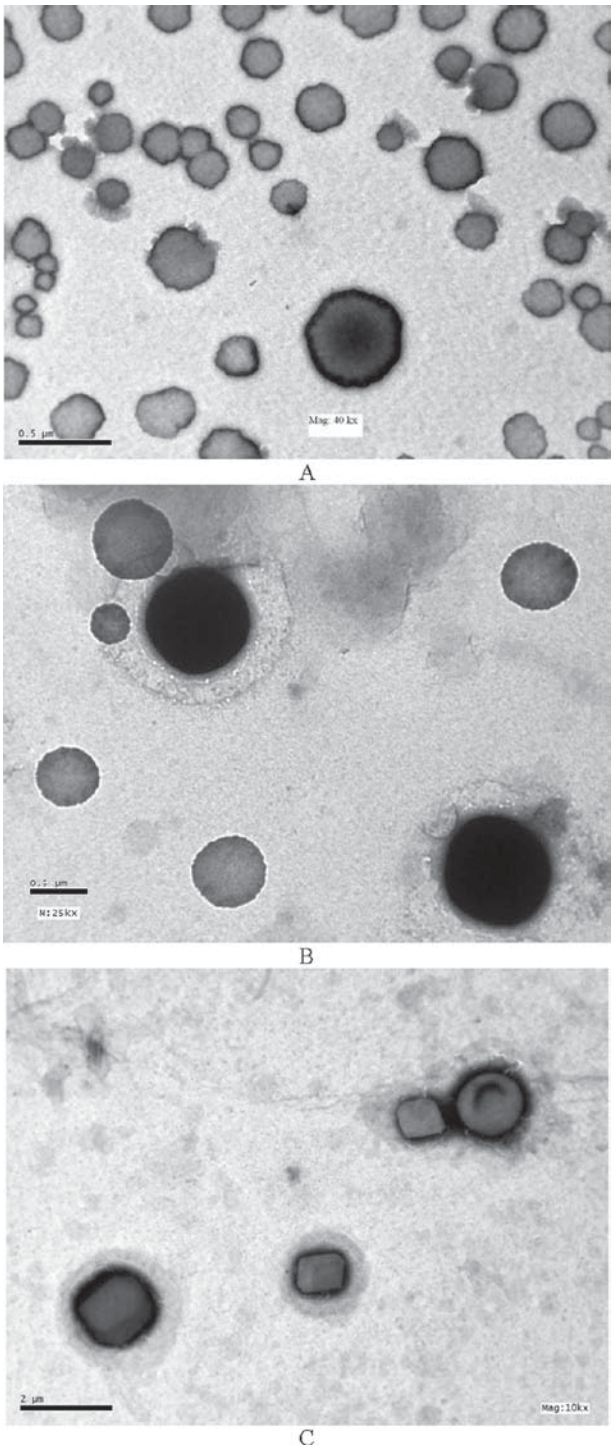


Figure 1. TEM micrographs of selected nystatin niosomes stained with 1 % phosphotungstic acid at different magnifications. (A) F_8 at 40 kx, (B) F_2 at 25 kx and (C) F_7 at 10 kx.

CHOL would be more likely to increase the number of bilayers because it has little effect on the charge at the bilayer surface and interbilayer separation[67].

Effect of NIS structure

It was also observed (Table 2) that Span 60 NYS niosomes, F_1–F_6, of the molar ratios NIS/CHOL/CIA (1:1:0.1 and 2:1:0.25) possessed larger vesicle sizes compared with Span 40 NYS niosomes, F_7–F_{12}, of the corresponding molar ratios. These findings agree with the fact, previously reported, that surfactants with longer alkyl chains and lower HLB generally form large vesicles[11,33,49,57,59–61,64,65,73], Span 60 (C_{18} and HLB = 4.7) and Span 40 (C_{16} and HLB = 6.7).

Effect of surface charge

Table 2 reveals that inclusion of DCP or SA in the niosomal formulations of the molar ratio NIS/CHOL/CIA (1:1:0.1) F_2, F_3, F_8 and F_9 decreased the vesicle size compared with neutral niosomes, F_1 and F_7. This might be

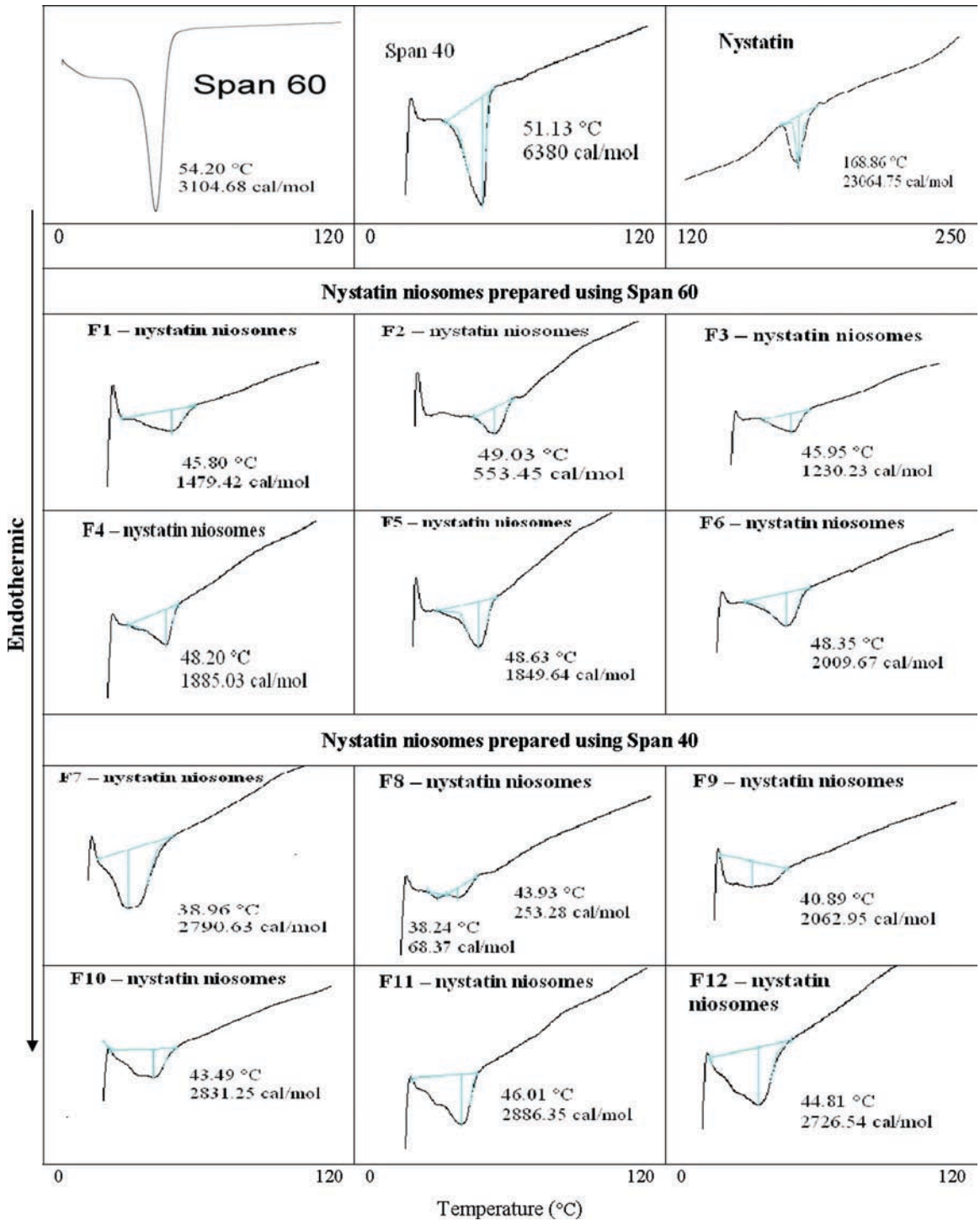


Figure 2. DSC thermograms of pure Span 60, Span 40 and nystatin in addition to F_1–F_{12} nystatin niosomes.

due to disproportionate distribution of DCP or SA in the bilayers, which may increase the curvature of the bilayer through the effect on electrostatic repulsion between the ionized head group, thus increasing the hydrophilic surface area. This effect will result in smaller vesicles[14,60,67,73–78]. On the contrary, inclusion of DCP or SA in the niosomal formulations of the molar ratio NIS/CHOL/CIA (2:1:0.25) F_5, F_6, F_{11} and F_{12}, respectively, increased the vesicle size compared with neutral vesicles, F_4 and F_{10}. This could be explained by the lower CHOL content (30.8 mole%) and higher CIA content (7.69 mole%) at molar ratio NIS/CHOL/CIA (2:1:0.25) compared with molar ratio NIS/CHOL/CIA (1:1:0.1) with CHOL content of 47.6 mole% and CIA content of 4.76 mole%, where the higher CIA

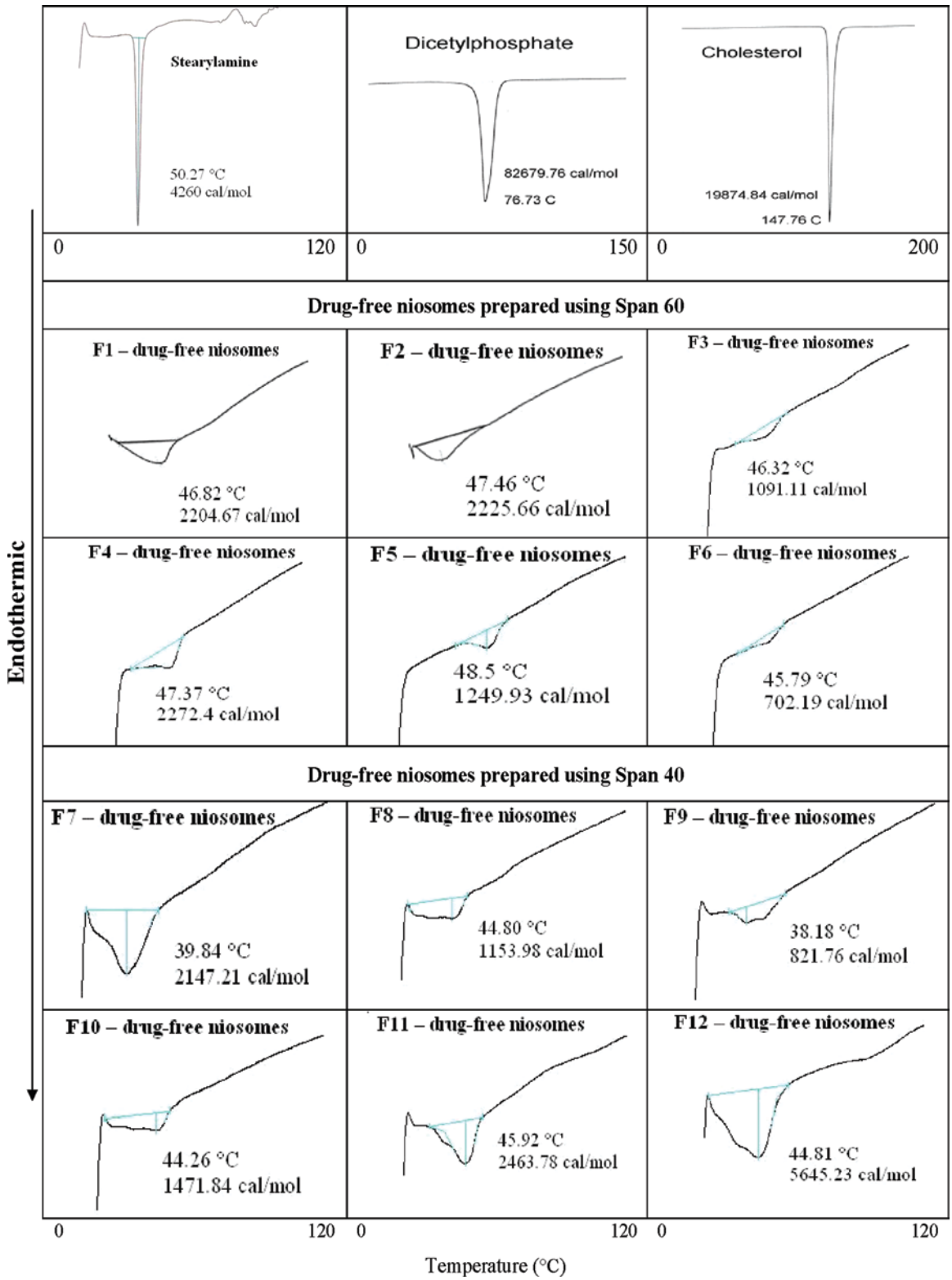


Figure 3. DSC thermograms of pure SA, DCP and CHOL in addition to F_1–F_{12} drug-free niosomes.

content combined with the lower CHOL content might have increased the hydrophilicity of the bilayers, thereby increasing the water intake of the bilayers of the vesicles, which resulted in increased vesicle size[14,35,60,79,80].

Polydispersity index

To investigate the width and homogeneity of the vesicle size distribution, PI was calculated according to Vora et al.[81] Table 2 reveals that the PI values of all niosomal formulations were <0.3, thus indicate a homogenous vesicular population[36,37]. A PI of 1 indicates large variations in vesicle size; a reported value of 0 means that size variation is absent[82]. The obtained low values of PI of the prepared niosomes indicate a limited variation in vesicle size.

In vitro *release profile studies of NYS niosomes*

Considering the release pattern for Span 60 or Span 40 NYS niosomes (Figure 4), it was observed that neutral NYS niosomes, F_1, F_4, F_7 and F_{10}, of both molar ratios NIS/CHOL (1:1 and 2:1) exhibited least drug release compared with negatively charged NYS niosomes, F_2, F_5, F_8 and F_{11}. The drug release occurred in two phases, an initial fast release that lasted for 3–6 h, followed by a sustained, but reduced, slow release that was maintained at least for 72 h (Figure 4). This release pattern was in agreement with that reported in previous works[30,32,67,74,83]. The biphasic release pattern might be due to size heterogeneity of the vesicles[84]. The rapid initial phase may be originated from permeation of free unentrapped NYS and desorption of drug from the surface of niosomes and the slower phase related primarily to the diffusion of NYS gradually through the bilayers into the medium[67,74,83]. Almost the entire amount of loaded drug was not released from the niosomes. This may be due to entrapment of the drug in the lipophilic region[50]. The slow release from the formulations might be beneficial for reducing the toxic side effects of NYS *in vivo*[65]. By reviewing the data in Table 2, $Q_{72\,h}$ for the niosomal formulations can be arranged in the following decreasing order: $F_{11} > F_5 > F_8 > F_2 > F_7 > F_{10} > F_1 > F_4$ niosomal vesicles. Differences in the *in vitro* release profiles may be due to vesicle size, lamellarity and membrane fluidity as a function of chain length of surfactant and CHOL content[85].

Effect of molar ratio (CHOL content) and surface charge

The results (Figure 4) show that neutral NYS niosomes of the molar ratio NIS/CHOL (1:1 or 2:1) revealed nearly similar release patterns comparing the relevant molar ratios, i.e. F_1 vs. F_4 and F_7 vs. F_{10}. This could be attributed to the high hydrophobic nature of NYS and its water insolubility; hence, the drug molecule is interchelated in the lipophilic region[50]. The CHOL content (50 and 33.33 mole%, respectively) had consequently a negligible effect on the leakage of drug from neutral niosomes. Although negatively charged NYS niosomes of the molar ratio NIS/CHOL/DCP (1:1:0.1 or 2:1:0.25) showed an increase of CHOL content in negatively charged niosomes, it markedly reduced the efflux of NYS from niosomal preparations, F_2 vs. F_5 and F_8 vs. F_{11}. CHOL stabilizes the bilayers, prevents leakiness and retards permeation of solutes enclosed in the vesicles[49,86]. CHOL is known to abolish the gel to liquid phase transition of niosomal systems and prevents leakage of drug from niosomes[58,87]. Cocera et al[88]. reported that CHOL produced an optimum hydrophobicity, which decreased the formation of the transient hydrophilic holes, by decreasing membrane fluidity, responsible for drug release through the bilayers.

However, negatively charged niosomes delivered more NYS as a result of electrostatic repulsion between bilayers and negatively charged NYS molecule. Increasing the DCP content in the molar ratio

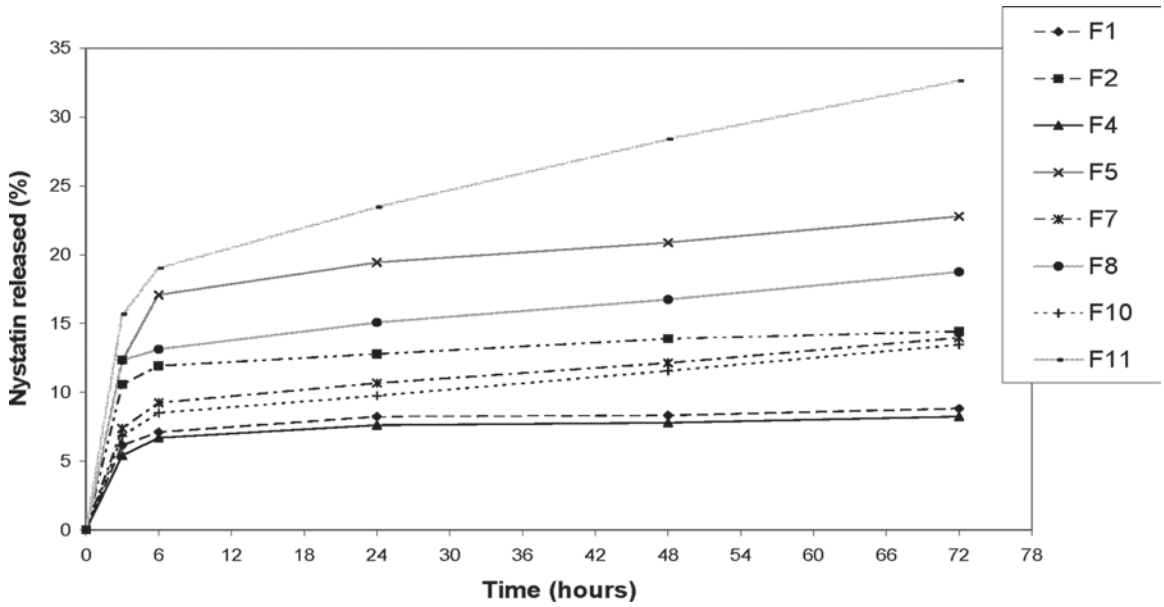


Figure 4. *In vitro* release profile of neutral and negatively charged nystatin niosomes.

Table 3. The calculated correlation coefficients and kinetics parameters of nystatin release profile from different niosomal formulations.

Formula	R^2 Value*				Order of Release	$K^{\dagger}$
	Zero Order	First Order	Second Order	Higuchi Model		
F_1	0.7671	0.7336	0.6988	0.8763	Higuchi	0.3527
F_2	0.8777	0.8469	0.8131	0.9443	Higuchi	0.5225
F_4	0.7277	0.6808	0.6335	0.8385	Higuchi	0.3584
F_5	0.7858	0.7094	0.6316	0.8722	Higuchi	1.3068
F_7	0.9342	0.8809	0.8119	0.9649	Higuchi	0.8696
F_8	0.9847	0.9692	0.9474	0.9923	Higuchi	0.9105
F_{10}	0.9582	0.9130	0.8478	0.9712	Higuchi	0.8867
F_{11}	0.9675	0.9225	0.8591	0.9909	Higuchi	2.3787

*R^2, correlation coefficient.
†K, release rate constant of Higuchi model (mg $h^{-1/2}$).

(1:1:0.1, DCP content = 4.76 mole% to 2:1:0.25, DCP content = 7.69 mole%) obviously increased the efflux of NYS through the niosomal vesicle. Increment of the DCP content led to an increase in the enormity of the electrostatic repulsion between bilayers and negatively charged NYS molecule, thus delivering more NYS to the release medium.

Effect of NIS structure

Span 40 NYS niosomes, F_7, F_8, F_{10} and F_{11}, possessed higher release rate compared with Span 60 NYS niosomes, F_1, F_2, F_4 and F_5 (Table 3). This could be possibly clarified by the fact that niosomes exhibit an alkyl chain length–dependent release and that the higher the chain length, *viz.* Span 60 (C_{18}) compared with Span 40 (C_{16}), the lower the release rate[48,49,57,64,65].

Kinetics studies of the release data

Table 3 reveals that Span 60 and Span 40 NYS niosomes' release pattern is most fitted to diffusion-controlled mechanism (Higuchi model). These results pointed to sustained release characteristics with a Higuchi pattern of drug release, where niosomes act as reservoir system for continuous delivery of drug. This slow release pattern of entrapped drug may indicate the high stability of the investigated niosomal formulations[72].

Sterilization of NYS niosomes

Sterility testing

The results of sterility testing of irradiated NYS niosomes show that there is no growth in negative control group, indicating that the tubes containing the medium are well sterilized, whereas growth took place in the two positive control groups representing aerobic bacteria (*E. coli*) and anaerobic bacteria (*C. sporogenes*), thus proving the suitability and ability of the medium to support the growth of both aerobic and anaerobic types of bacteria. In tubes inoculated with the biological indicator, *B. pumilus* E601, growth was detected in the radiation doses of zero and 15 kGy, whereas no growth was observed at the radiation dose of 25 kGy, which is the radiation dose known to stop the viability of this microorganism. For tubes inoculated with NYS niosomal suspension, growth was observed at the doses zero and 15 kGy, whereas no growth was observed at the radiation dose of 25 kGy in the two NYS niosomal formulations investigated, indicating that the irradiation dose of 25 kGy is sufficient for accomplishing radiation sterilization of the product. These results indicate that the irradiation dose of 25 kGy is the optimum sterilization dose for NYS niosomal formulations investigated in this experiment.

Table 4. Effect of γ-irradiation on the stability of the niosomal formulations.

Radiation Dose (kGy)	EE% (Mean ± SD)*			
	F_1		F_{10}	
Zero (control)	96.28 ± 0.74		87.49 ± 1.11	
15	95.43 ± 1.02		86.64 ± 1.21	
25	93.07 ± 0.46		83.23 ± 1.39	
Radiation Dose (kGy)	P	Radiation Dose (kGy)		P
F_1		F_{10}		
Zero vs. 15	>0.05	Zero vs. 15		>0.05
Zero vs. 25	>0.05	Zero vs. 25		>0.05

*Mean of two determinations.

Effect of γ-irradiation on the stability of the formulation

Table 4 reveals that there was a nonsignificant difference ($P > 0.05$) between the EE% of F_1 and F_{10} NYS niosomes before and after the two irradiation doses, *viz.* 15 and 25 kGy. These findings indicate that γ-irradiation does not disrupt the vesicles, induce leakage of NYS from niosomal vesicles or deteriorate the drug molecules. The leakage of NYS from the niosomal vesicles was minimal and nonsignificant ($P > 0.05$) for the tested formulae.

In vivo renal and hepatic toxicity

Biochemical tests

Treatment with NYS niosomes was found to exert less nephrotoxicity and hepatotoxicity compared with free NYS (Figure 5). Estimations of AST/sGOT activities revealed a nonsignificant difference between all treatment groups and controls ($P > 0.05$), whereas estimations of ALT/sGPT activities showed a significant

difference ($P < 0.05$) between the group receiving drug-free niosomes and the group receiving free NYS. The same results ($P < 0.05$) were observed between the two groups receiving F_1–NYS niosomes or F_{10}–NYS niosomes compared with the group receiving free NYS. For BUN content estimations, there was a significant difference ($P < 0.05$) between the group receiving free NYS and the group receiving drug-free niosomes, and no significant difference ($P > 0.05$) between the other groups was observed. Serum creatinine content estimations showed a significant difference ($P < 0.05$) between free NYS group and both drug-free niosomes and control group, DMSO/saline (1:1 vol/vol). Furthermore, there was a significant difference ($P < 0.05$) between the group receiving free NYS and the group receiving F_1–NYS niosomes.

Histopathologic studies

Histopathologic studies were performed on livers and kidneys isolated from two rats of each group. The organs were stained with hematoxylin and eosin stain and examined for any histopathologic findings. Figure 6 shows the images of the livers and kidneys of the treated rats. According to micrographs, niosomal

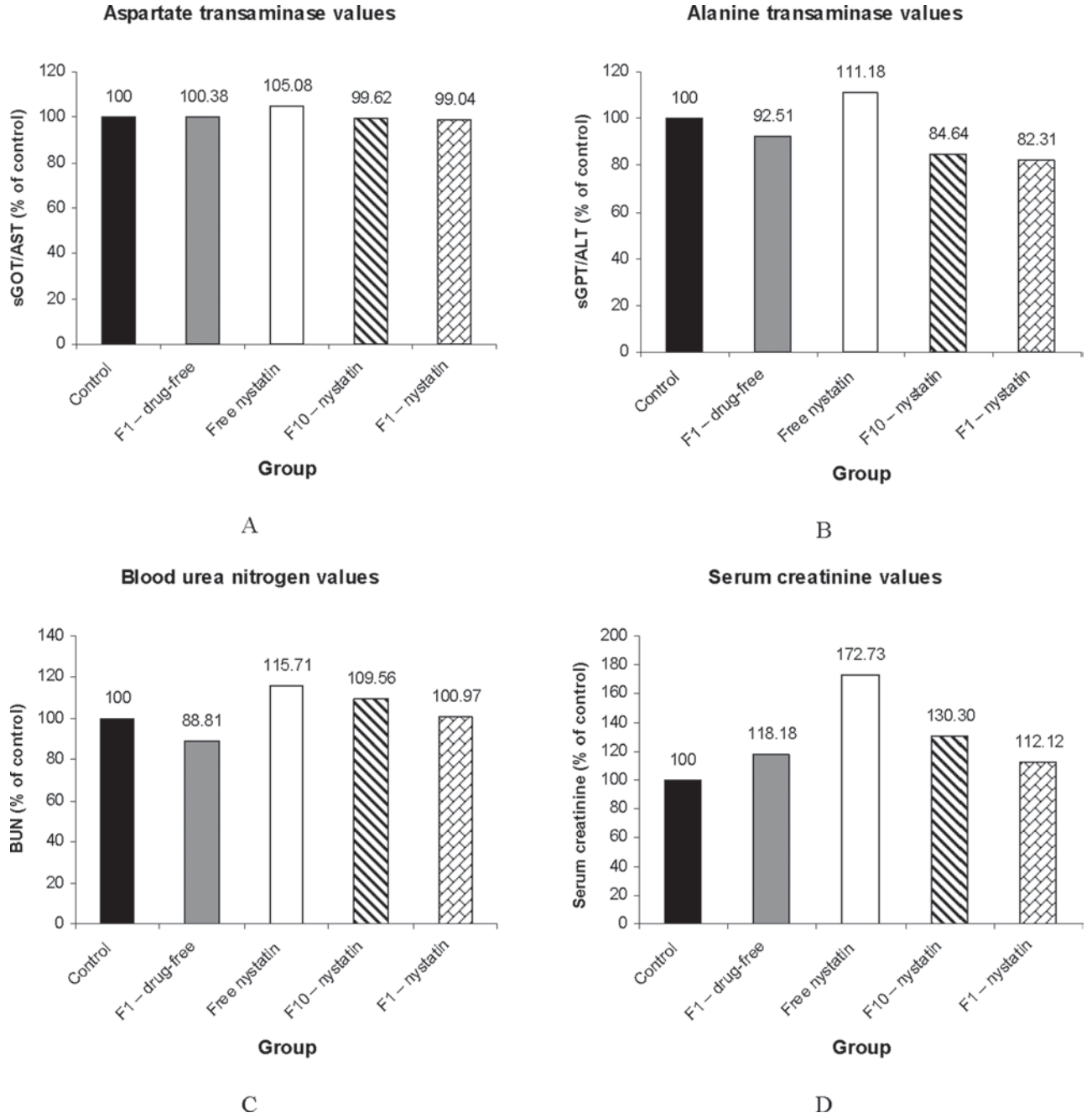
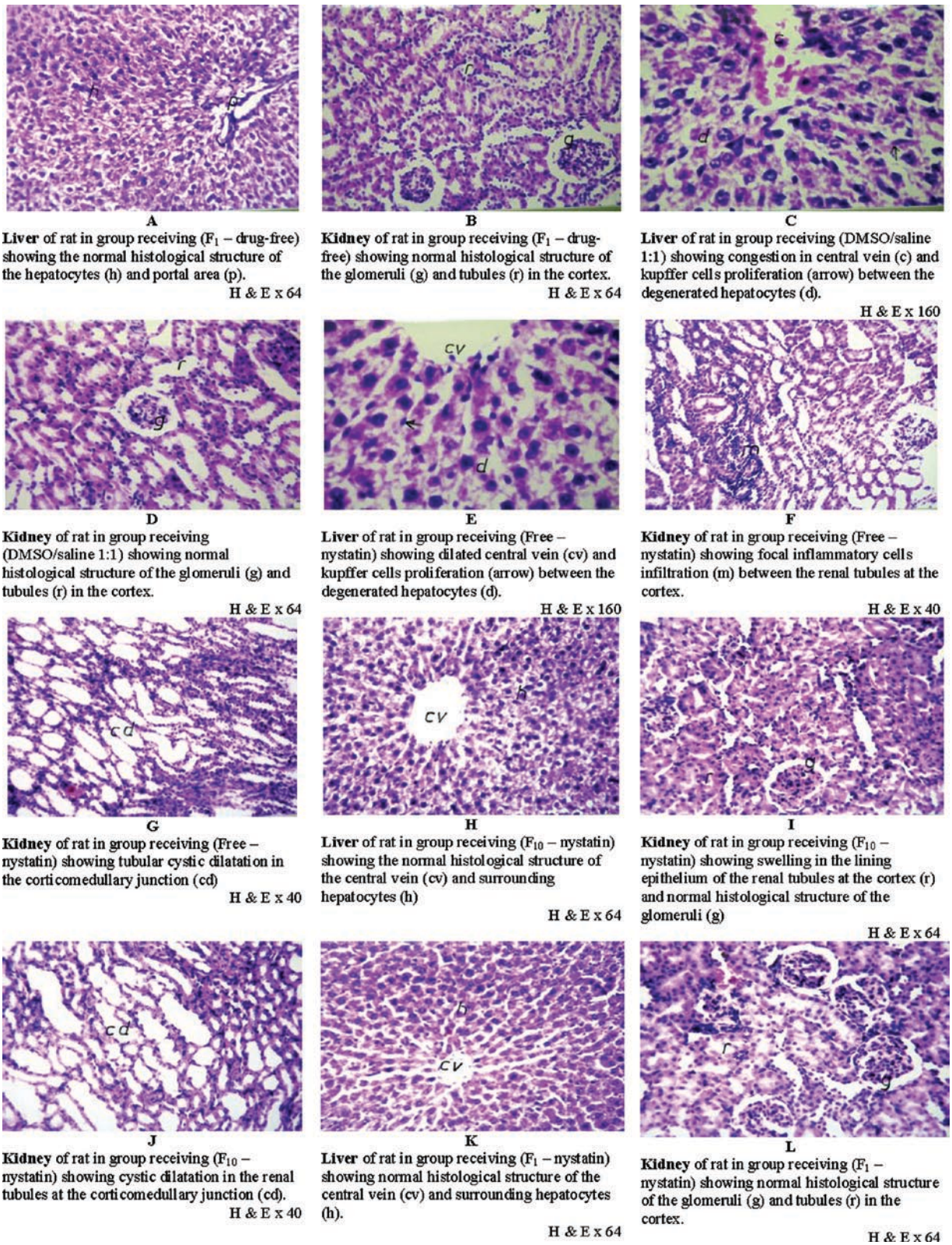


Figure 5. Serum transaminases (A and B), blood urea nitrogen (C) and creatinine (D) values (percentage of control) of rats treated with nystatin (5 mg/kg) for 5 consecutive days. Data presented as percentage of control = (biochemical value of treatment group/biochemical value of control group) × 100.

NYS shows no pathologic abnormalities compared with free NYS with distinct alterations. Control groups are also shown.

The former biochemical and histopathologic findings reveal that the incorporation of NYS into niosomes led to a decrease in the reported toxicity associated with its free form, when administered parenterally. These results emphasize the safety of the formulation. This could be attributed to the slow release from the formulations, which might reduce the toxic side effects of NYS *in vivo*[65] and the targeting of vesicular systems to organs of extravasation, thus decreasing toxicity. The results also



A
Liver of rat in group receiving (F_1 – drug-free) showing the normal histological structure of the hepatocytes (h) and portal area (p).
H & E x 64

B
Kidney of rat in group receiving (F_1 – drug-free) showing normal histological structure of the glomeruli (g) and tubules (r) in the cortex.
H & E x 64

C
Liver of rat in group receiving (DMSO/saline 1:1) showing congestion in central vein (c) and kupffer cells proliferation (arrow) between the degenerated hepatocytes (d).
H & E x 160

D
Kidney of rat in group receiving (DMSO/saline 1:1) showing normal histological structure of the glomeruli (g) and tubules (r) in the cortex.
H & E x 64

E
Liver of rat in group receiving (Free – nystatin) showing dilated central vein (cv) and kupffer cells proliferation (arrow) between the degenerated hepatocytes (d).
H & E x 160

F
Kidney of rat in group receiving (Free – nystatin) showing focal inflammatory cells infiltration (m) between the renal tubules at the cortex.
H & E x 40

G
Kidney of rat in group receiving (Free – nystatin) showing tubular cystic dilatation in the corticomedullary junction (cd)
H & E x 40

H
Liver of rat in group receiving (F_{10} – nystatin) showing the normal histological structure of the central vein (cv) and surrounding hepatocytes (h)
H & E x 64

I
Kidney of rat in group receiving (F_{10} – nystatin) showing swelling in the lining epithelium of the renal tubules at the cortex (r) and normal histological structure of the glomeruli (g)
H & E x 64

J
Kidney of rat in group receiving (F_{10} – nystatin) showing cystic dilatation in the renal tubules at the corticomedullary junction (cd).
H & E x 40

K
Liver of rat in group receiving (F_1 – nystatin) showing normal histological structure of the central vein (cv) and surrounding hepatocytes (h).
H & E x 64

L
Kidney of rat in group receiving (F_1 – nystatin) showing normal histological structure of the glomeruli (g) and tubules (r) in the cortex.
H & E x 64

Figure 6. Histopathologic micrographs of rats' livers and kidneys on day 6 post 5 days treatment with nystatin (5 mg/kg).

show that Span 60 niosomes showed higher safety compared with Span 40 vesicles, which could be explained by the fact that the increase in alkyl chain length is accompanied by the decrease in toxicity[16,89]. According to the biochemical values, mice treated with free NYS showed higher nephrotoxicity compared with the other treatment groups. Polyene antibiotics have been found to bind with low-density lipoproteins (LDL), and high numbers of renal LDL receptors mediate the cellular uptake of these drugs[90,91]. This association is believed to be a major contributor to the dose-limiting nephrotoxicity of NYS[2]. According to literature, liposomal and niosomal formulations have a high affinity for binding to high-density lipoproteins (HDL[2]). Binding to HDL promotes uptakes in the reticuloendothelial system (RES), which has a relatively high level of expression of HDL receptors[90]. This seems one of the reasons of less nephrotoxicity of amphotericin B and NYS upon incorporation into liposomes or niosomes[2].Thus, the preferential uptake of niosomal NYS by hepatic and splenic macrophages lead to reduced availability of the drug to the kidney, which ultimately helps in less nephrotoxicity and improved therapeutic index[2].

Various liposomal formulations of polyene antifungal drugs, *viz.* amphotericin B and NYS, have been found to impart cure against most common fungal pathogens[92]. Hence, the liposomal or niosomal formulations increase the therapeutic index of the drug by delivering higher medicament concentrations to the infected tissues and therefore reducing the toxicity of the drug to the normal cells[24,92].

Tissue distribution study

The results in Figure 7 demonstrate the levels of NYS in the liver, spleen and lungs of mice after a single dose of nystatin (5 mg/kg) injected intraperitoneally. Levels were determined at 24 and 48 h postinjection. Drug concentrations in tissues were calculated to 1 g of tissue. The highest concentrations were shown in the spleen > liver > lungs after 24 and 48 h. F_1–NYS niosomes were leading all over the experiment, showing higher levels of NYS in the different organs followed by F_{10}–NYS niosomes and the lowest levels possessed by the free form. Niosomal formulations showed a significant difference ($P < 0.05$) compared with free NYS. These findings reveal the superiority of niosomes to dispose higher amounts of drug by successful targeting to vital organs. In fact, the results showed that a major fraction of the drug is found in the liver and spleen, i.e. organs rich in RES, when the drug is administered as a niosomal formulation. The niosome-mediated passive targeting of NYS enables the drug to be accumulated in these organs[2]. This may be explained on the basis that liposomal and niosomal formulations have a high affinity for binding to HDL[90]. Binding to HDL promotes uptakes in RES, which has a relatively high level of expression of HDL receptors[90].

Antifungal efficacy

In this study, the efficacy of NYS niosomes compared with free NYS in the elimination of *C. albicans* infection from tissues was assessed by quantification of fungal burden (day 6 postinfection) in vital organs, i.e. lungs, spleen, liver, kidney and heart. A remarkable reduction in fungal load was observed in the organs of mice treated with NYS entrapped in niosomes (Table 5; Figure 8). NYS niosomes revealed significantly higher activity in eliminating the pathogen from tissues compared with free NYS or controls ($P < 0.05$). The group receiving F_1–NYS niosomes showed least CFU counts followed by F_{10}–NYS niosomes with nonsignificant difference ($P > 0.05$). Both formulations had a pronounced reduction of fungal burden ($P < 0.05$) compared with free NYS and control groups.

It was reported that fungal infections are mainly confined to the immunocompromised subjects[20]; the study was performed in neutropenic animal models. On administration into the host, the niosomes are avidly taken up by macrophages, which subsequently act as secondary depot and help in carrying the active drug molecules to the site of infection[93].

The results obtained demonstrate that after incorporation into niosomes, NYS became an active therapeutic

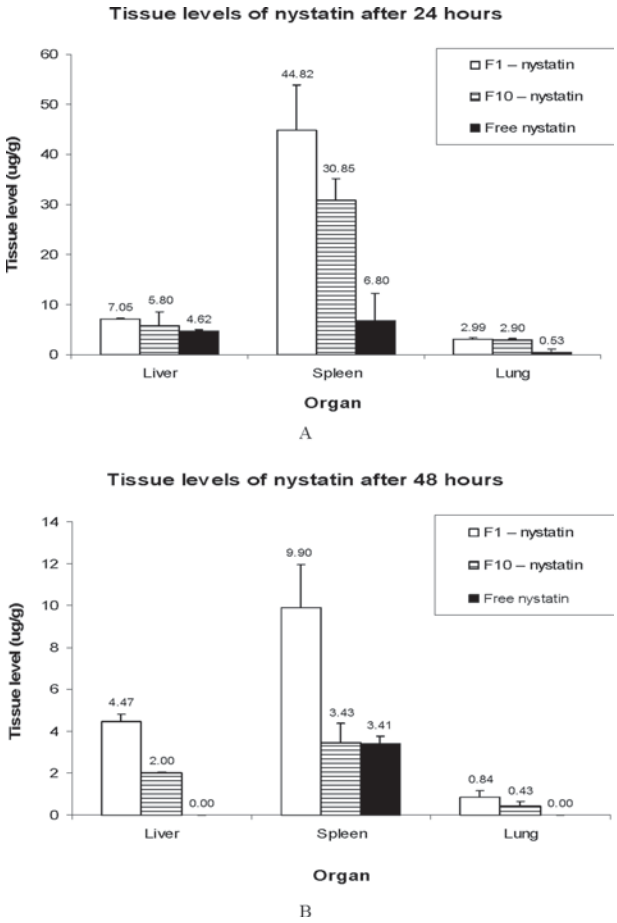


Figure 7. Tissue distribution levels (μg/g) after 24 (A) and 48 (B) h followed by a single dose of nystatin (5 mg/kg). Data are presented as mean ± SD.

Table 5. Percentage reduction of fungal load in vital organs after 5-day treatment regimen with nystatin (5 mg/kg) in infected mice with *Candida albicans*

	(% Reduction/g of Organ)* Mean† ± SD % on Day 6 Postinfection				
Groups	Liver	Spleen	Lung	Kidney	Heart
F_1–nystatin	92.26%	90.75%	93.34%	78.66%	42.34%
F_{10}–nystatin	89.46%	85.49%	89.42%	77.94 %	36.02%
Free nystatin	26.61%	30.74%	86.58%	17.59%	23.24%
F_1–drug-free niosomes	–23.66%	–38.07%	–52.81%	–24.73%	–15.07%
DMSO/saline 1:1 (vol/vol) (control)	—	—	—	—	—

*Percent reduction is expressed as (mean CFU/g of control group – mean CFU/g of treatment group) × 100/mean CFU/g of control group.
†Mean of three animals.

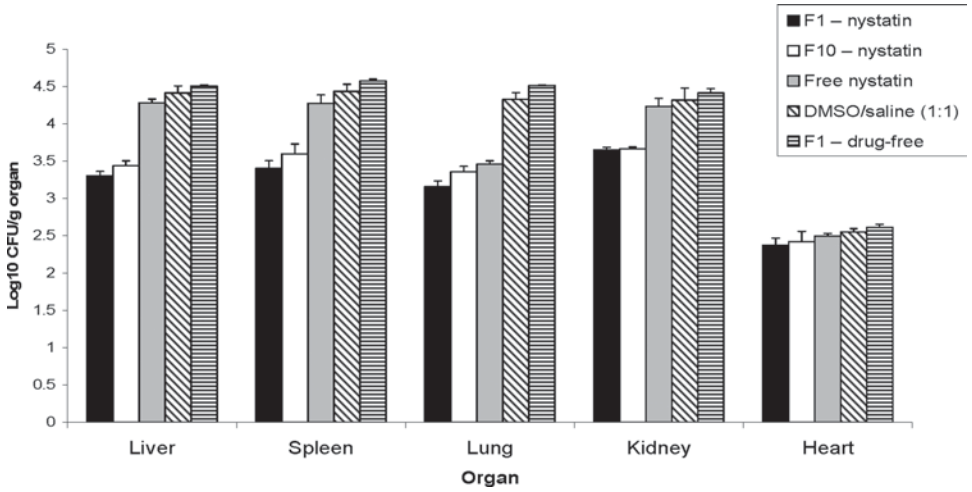


Figure 8. Comparative efficacy of different treatments against *Candida albicans* infection in mice after five daily doses of nystatin (5 mg/ kg). Data are presented as mean $\log_{10}$ CFU/g ± SD.

agent in the treatment of systemic experimental candidiasis. Although free NYS had significant antifungal activity *in vitro*, it was toxic and noneffective when administered intravenously[5]. Niosomal encapsulation provided an injectable formulation of NYS, which was observed to be significantly less toxic than the free drug. Niosomal NYS was less toxic (5 mg/kg) than the free NYS (5 mg/kg) as observed in the toxicity study. The improvement of the therapeutic treatment against *C. albicans* was to some extent related to the reduced toxicity of the drug after encapsulation in niosomes[5], where the reported side effects are reduced, and it is possible to administer the therapeutic dose safely. The observed ineffectiveness of free NYS as a systemic antifungal may be due, in part, to inadequate delivery of the drug to affected sites[5]. Niosomal encapsulation allowed the systemic administration of NYS and its use as an effective antifungal agent in mice. The results show that niosomal incorporation of NYS provides an effective and well-tolerated therapy in a neutropenic mouse model of disseminated *Candida* infection.

The high tissue level and slower release rate of NYS niosomes provides sufficient time for the drug to remain in the circulation and in contact with the pathogen, which leads to the eradication and elimination of the pathogen from circulation[20].

Conclusion

The results of this study show that CHOL content, type of surfactant and the presence of CIA altered the EE%, vesicle size and release rate of NYS niosomes. Neutral and positively charged niosomes showed the highest entrapment percentage of NYS. NYS release from niosomes was low, most fitted to Higuchi diffusion mathematical model. The biological evaluation of NYS niosomes on animal model exerted less nephrotoxicity and hepatotoxicity, showed higher level of drug in vital organs and revealed privilege efficacy in elimination of the fungal burden compared with those treated with free NYS. From the observations made in the current study, it could be inferred that NYS niosomes had extra advantages over free NYS because of reduced toxicity, higher tissue distribution and improved efficacy. Therefore, the findings of increased activity of antifungal agents by encapsulation into niosomes may be of great clinical significance in cases of immunocompromised persons, the main sufferer of fungal infections.

Declaration of interest

The authors report no declarations of interest.

References

1. Kauffman CA. (2006). Clinical efficacy of new antifungal agents. Curr Opin Microbiol, 9:483–488.
2. Khan MA, Faisal SM, Mohammad O. (2006). Safety, efficacy and pharmacokinetics of tuftsin-loaded nystatin liposomes in murine model. J Drug Target, 14:233–241.
3. Pace HR, Schantz SI. (1956). Nystatin (mycostatin) in the treatment of monilial and nonmonilial vaginitis. J Am Med Assoc, 162:268–271.
4. Ng AW, Wasan KM, Lopez-Berestein G. (2003). Development of liposomal polyene antibiotics: an historical perspective. J Pharm Pharm Sci, 6:67–83.
5. Mehta RT, Hopfer RL, McQueen T, Juliano RL, Lopez-Berestein G. (1987). Toxicity and therapeutic effects in mice of liposome-encapsulated nystatin for systemic fungal infections. Antimicrob Agents Chemother, 31:1901–1903.
6. Wallace TL, Paetznick V, Cossum PA, Lopez-Berestein G, Rex JH, Anaissie E. (1997). Activity of liposomal nystatin against disseminated Aspergillus fumigatus infection in neutropenic mice. Antimicrob Agents Chemother, 41:2238–2243.
7. Frokjaer S, Hjorth EL, Worts O. Stability testing of liposomes during storage. In Liposome Technology: Preparation of Liposomes, 1st edn., Gregoriadis, G. (ed.), Vol. 1, (1984), CRC Press Inc, Boca Raton, FL, pp. 235–245.
8. Arien A, Dupuy B. (1997). Encapsulation of calcitonin in liposomes depends on the vesicle preparation method. J Microencapsul, 14:753–760.
9. Alsarra IA, Bosela AA, Ahmed SM, Mahrous GM. (2005). Proniosomes as a drug carrier for transdermal delivery of ketorolac. Eur J Pharm Biopharm, 59:485–490.
10. Azmin MN, Florence AT, Handjani-Vila RM, Stuart JF, Vanlerberghe G, Whittaker JS. (1985). The effect of non-ionic surfactant vesicle (niosome) entrapment on the absorption and distribution of methotrexate in mice. J Pharm Pharmacol, 37:237–242.
11. Uchegbu IF, Vyas SP. (1998). Non-ionic surfactant based vesicles (niosomes) in drug delivery. Int J Pharm, 172:33–70.
12. Uchegbu IF, Florence AT. (1995). Non-ionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry. Adv Colloid Interface Sci, 58:1–55.
13. Arora R, Jain CP. (2007). Advances in niosome as a drug carrier: A review. Asian J Pharm, 1: 29–39.
14. Junyaprasert VB, Teeranachaideekul V, Supaperm T. (2008). Effect of charged and non-ionic membrane additives on physicochemical properties and stability of niosomes. AAPS Pharmscitech, 9:851–859.
15. Palozza P, Muzzalupo R, Trombino S, Valdannini A, Picci N. (2006). Solubilization and stabilization of beta-carotene in niosomes: delivery to cultured cells. Chem Phys Lipids, 139:32–42.
16. Azeem A, Anwer MK, Talegaonkar S. (2009). Niosomes in sustained and targeted drug delivery: some recent advances. J Drug Target, 17:671–689.
17. Bangham AD, Standish MM, Watkins JC. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol, 13:238–252.
18. Baillie AJ, Florence AT, Hume LR, Muirhead GT, Rogerson A. (1985). The preparation and properties of niosomes–non-ionic surfactant vesicles. J Pharm Pharmacol, 37:863–868.
19. Khan MA, Firoz A, Jabeen R, Mohammad O. (2004). Prophylactic role of immunomodulators in treatment of systemic candidiasis in leukopenic mice. J Drug Target, 12:425–433.
20. Khan MA, Nasti TH, Saima K, Mallick AI, Firoz A, Wajahul H et al. (2004). Co-administration of immunomodulator tuftsin and liposomised nystatin can combat less susceptible Candida albicans infection in temporarily neutropenic mice. FEMS Immunol Med Microbiol, 41:249–258.
21. Khan MA, Syed FM, Nasti HT, Saima Dagger K, Haq W, Shehbaz A et al. (2003). Use of tuftsin bearing nystatin liposomes against an isolate of Candida albicans showing less *in vivo* susceptibility to amphotericin B. j Drug Target, 11:93–99.
22. Moribe K, Maruyama K, Iwatsuru M. (2000). Spectroscopic investigation of the molecular state of nystatin encapsulated in liposomes. Int J Pharm, 201:37–49.
23. Moribe K, Maruyama K, Iwatsuru M. (1999). Encapsulation characteristics of nystatin in liposomes: effects of cholesterol and polyethylene glycol derivatives. Int J Pharm, 188:193–202.
24. Nasti TH, Khan MA, Owais M. (2006). Enhanced efficacy of pH-sensitive nystatin liposomes against Cryptococcus neoformans in murine model. J Antimicrob Chemother, 57:349–352.
25. Cassidy SM, Strobel FW, Wasan KM. (1998). Plasma lipoprotein distribution of liposomal nystatin is influenced by protein content of high-density lipoproteins. Antimicrob Agents Chemother, 42:1878–1888.
26. Wasan KM, Ramaswamy M, Cassidy SM, Kazemi M, Strobel FW, Thies RL. (1997). Physical characteristics and lipoprotein distribution of liposomal nystatin in human plasma. Antimicrob Agents Chemother, 41:1871–1875.
27. Mehta RT, Hopfer RL, Gunner LA, Juliano RL, Lopez-Berestein G. (1987). Formulation, toxicity, and antifungal activity *in vitro* of liposome-encapsulated nystatin as therapeutic agent for systemic candidiasis. Antimicrob Agents Chemother, 31:1897–1900.
28. Hashim F, El-Ridy M, Nasr M, Abdallah Y. (2010). Preparation and characterization of niosomes containing ribavirin for liver targeting. Drug Deliv, 17:282–287.
29. El-Ridy MS, Mostafa DM, Shehab A, Nasr EA, Abd El-Alim S. (2007). Biological evaluation of pyrazinamide liposomes for treatment of *Mycobacterium tuberculosis*. Int J Pharm, 330:82–88.
30. Mokhtar M, Sammour OA, Hammad MA, Megrab NA. (2008). Effect of some formulation parameters on flurbiprofen encapsulation and release rates of niosomes prepared from proniosomes. Int J Pharm, 361:104–111.
31. Nystatin. In: British Pharmacopoeia, vol. II, (2007), pp. 1497–1498.
32. Hong M, Zhu S, Jiang Y, Tang G, Pei Y. (2009). Efficient tumor targeting of hydroxycamptothecin loaded PEGylated niosomes modified with transferrin. J Control Release, 133:96–102.
33. Di Marzio L, Marianecci C, Petrone M, Rinaldi F, Carafa M. (2011). Novel pH-sensitive non-ionic surfactant vesicles: comparison between Tween 21 and Tween 20. Colloids Surf B Biointerfaces, 82:18–24.
34. Marianecci C, Paolino D, Celia C, Fresta M, Carafa M, Alhaique F. (2010). Non-ionic surfactant vesicles in pulmonary glucocorticoid delivery: characterization and interaction with human lung fibroblasts. J Control Release, 147:127–135.
35. Carafa M, Santucci E, Lucania G. (2002). Lidocaine-loaded non-ionic surfactant vesicles: characterization and *in vitro* permeation studies. Int J Pharm, 231:21–32.
36. Sentjurc M, Vrhovnik K, Kristl J. (1999). Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method. J Control Release, 59:87–97.
37. Centis V, Vermette P. (2008). Physico-chemical properties and cytotoxicity assessment of PEG-modified liposomes containing human hemoglobin. Colloids Surf B Biointerfaces, 65:239–246.
38. Juliano RL, Stamp D. (1978). Pharmacokinetics of liposome-encapsulated anti-tumor drugs. Studies with vinblastine, actinomycin D, cytosine arabinoside, and daunomycin. Biochem Pharmacol, 27:21–27.
39. El-Ridy MS, Kassem M, Akbarieh M, Tawashi R. (1988). The effect of surface charge of liposomes on aggregation in the buccal cavity. In: Proceedings 15th Int. Symp. Control. Rel. Bioact. Mater. Basel, Switzerland.
40. Parisi AN, Antoine AD. (1975). Characterization of Bacillus pumilus E601 spores after single sublethal gamma irradiation treatments. Appl Microbiol, 29:34–39.
41. Gamble M. (1996). The hematosylins and eosin. In: Bancroft JD, Stevens A, Turner DR (ed.), Theory and Practice of Histological Techniques, 4th edn. Churchill Livingstone, New York, Edinburgh, London, Madrid, Melbourne, San Francisco, Tokyo, pp. 99–112.

42. Groll AH, Mickiene D, Werner K, Petraitiene R, Petraitis V, Calendario M et al. (2000). Compartmental pharmacokinetics and tissue distribution of multilamellar liposomal nystatin in rabbits. Antimicrob Agents Chemother, 44:950–957.
43. Groll AH, Mickiene D, Werner K, Piscitelli SC, Walsh TJ. (1999). High-performance liquid chromatographic determination of liposomal nystatin in plasma and tissues for pharmacokinetic and tissue distribution studies. J Chromatogr B Biomed Sci Appl, 735:51–62.
44. Groll AH, Mickiene D, Petraitis V, Petraitiene R, Alfaro RM, King C et al. (2003). Comparative drug disposition, urinary pharmacokinetics, and renal effects of multilamellar liposomal nystatin and amphotericin B deoxycholate in rabbits. Antimicrob Agents Chemother, 47:3917–3925.
45. Shalit I, Horev-Azaria L, Fabian I, Blau H, Kariv N, Shechtman I et al. (2002). Immunomodulatory and protective effects of moxifloxacin against Candida albicans-induced bronchopneumonia in mice injected with cyclophosphamide. Antimicrob Agents Chemother, 46:2442–2449.
46. Denning DW, Warn P. (1999). Dose range evaluation of liposomal nystatin and comparisons with amphotericin B and amphotericin B lipid complex in temporarily neutropenic mice infected with an isolate of Aspergillus fumigatus with reduced susceptibility to amphotericin B. Antimicrob Agents Chemother, 43:2592–2599.
47. Shi B, Fang C, Pei Y. (2006). Stealth PEG-PHDCA niosomes: effects of chain length of PEG and particle size on niosomes surface properties, *in vitro* drug release, phagocytic uptake, *in vivo* pharmacokinetics and antitumor activity. J Pharm Sci, 95:1873–1887.
48. Devaraj GN, Parakh SR, Devraj R, Apte SS, Rao BR, Rambhau D. (2002). Release studies on niosomes containing fatty alcohols as bilayer stabilizers instead of cholesterol. J Colloid Interface Sci, 251:360–365.
49. Abdelbary G, El-Gendy N. (2008). Niosome-encapsulated gentamicin for ophthalmic controlled delivery. AAPS Pharmscitech, 9:740–747.
50. Ruckmani K, Sankar V. (2010). Formulation and optimization of Zidovudine niosomes. AAPS Pharmscitech, 11:1119–1127.
51. Gianasi E, Cociancich F, Uchegbu IF, Florence AT, Duncan R. (1997). Pharmaceutical and biological characterization of a doxorubicin polymer conjugate (PK1) entrapped in sorbitan monostearate Span 60 niosomes. Int J Pharm, 148:139–148.
52. Jain S, Umamaheswari B, Bhadra D, Jain NK. (2004). Ethosomes: a novel carrier for enhanced transdermal delivery of an anti-HIV agent. Indian J Pharm Sci, 66:72–81.
53. Hac-Wydro K, Dynarowicz-Latka P. (2006). Nystatin in Langmuir monolayers at the air/water interface. Colloids Surf B Biointerfaces, 53:64–71.
54. Hao YM, Li K. (2011). Entrapment and release difference resulting from hydrogen bonding interactions in niosome. Int J Pharm, 403:245–253.
55. Mohammed AR, Weston N, Coombes AG, Fitzgerald M, Perrie Y. (2004). Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. Int J Pharm, 285:23–34.
56. Yamamoto S, Nakano K, Ishikawa C, Yamamoto M, Matsumoto Y, Iwahara M, Furusaki S, Ueoka R. (2002). Enhanced inhibitory effects of extracts from *Ginkgo biloba* L. leaves encapsulated in hybrid liposomes on the growth of tumor cells *in vitro*. Biochem Eng J, 12:125–130.
57. Nasr M, Mansour S, Mortada ND, Elshamy AA. (2008). Vesicular aceclofenac systems: a comparative study between liposomes and niosomes. J Microencapsul, 25:499–512.
58. Hao Y, Zhao F, Li N, Yang Y, Li K. Studies on a high encapsulation of colchicine by a niosome system. Int J Pharm, 244:73–80.
59. Yoshioka T, Sternberg B, Florence AT. (1994). Preparation and properties of vesicles (niosomes) of sorbitan monoesters (Span 20, 40, 60 and 80) and a sorbitan triester (Span 85). Int J Pharm, 105:1–6.
60. Balakrishnan P, Shanmugam S, Lee WS, Lee WM, Kim JO, Oh DH et al. (2009). Formulation and *in vitro* assessment of minoxidil niosomes for enhanced skin delivery. Int J Pharm, 377:1–8.
61. Manosroi A, Wongtrakulc P, Manosroia J, Sakaid H, Sugawarad F, Yuasad M, Abed M. (2003). Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. Colloids Surf. B: Biointerfaces, 30:129–138.
62. RajaNaresh RA, Pillai GK, Udupa N, Chandrashekar G. (1994). Anti-inflammatory activity of niosome encapsulated diclofenac sodium in arthritic rats. Indian J Pharmacol, 26:46–48.
63. Manconi M, Sinico C, Valenti D, Loy G, Fadda AM. (2002). Niosomes as carriers for tretinoin. I. Preparation and properties. Int J Pharm, 234:237–248.
64. Guinedi AS, Mortada ND, Mansour S, Hathout RM. (2005). Preparation and evaluation of reverse-phase evaporation and multilamellar niosomes as ophthalmic carriers of acetazolamide. Int J Pharm, 306:71–82.
65. Bayindir ZS, Yuksel N. (2010). Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery. J Pharm Sci, 99:2049–2060.
66. Lawrence MJ, Chauhan S, Lawrence SM, Barlow DJ. (1996). The formation, characterization and stability of non-ionic surfactant vesicles. STP Pharm Sci, 6:49–60.
67. Varshosaz J, Pardakhty A, Hajhashemi VI, Najafabadi AR. (2003). Development and physical characterization of sorbitan monoester niosomes for insulin oral delivery. Drug Deliv, 10:251–262.
68. Fresta M, Puglisi G. (1997). Corticosteroid dermal delivery with skin-lipid liposomes. J Control Release, 44:141–151.
69. El Maghraby GM, Campbell M, Finnin BC. (2005). Mechanisms of action of novel skin penetration enhancers: phospholipid versus skin lipid liposomes. Int J Pharm, 305:90–104.
70. Moazeni E, Gilani K, Sotoudegan F, Pardakhty A, Najafabadi AR, Ghalandari R et al. (2010). Formulation and *in vitro* evaluation of ciprofloxacin containing niosomes for pulmonary delivery. J Microencapsul, 27:618–627.
71. Seras-Cansel A, Ollivon M, Lesieur S. (1996). Generation of non-ionic monoalkyl amphiphile-cholesterol vesicles: Evidence of membranen impermeability to octyl glycoside. STP Pharm Sci, 6:12–20.
72. Attia IA, El-Gizawy SA, Fouda MA, Donia AM. (2007). Influence of a niosomal formulation on the oral bioavailability of acyclovir in rabbits. AAPS Pharmscitech, 8:E106.
73. Pardakhty A, Varshosaz J, Rouholamini A. (2007). *In vitro* study of polyoxyethylene alkyl ether niosomes for delivery of insulin. Int J Pharm, 328:130–141.
74. Khazaeli P, Pardakhty A, Shoorabi H. (2007). Caffeine-loaded niosomes: characterization and *in vitro* release studies. Drug Deliv, 14:447–452.
75. Tabbakhian M, Tavakoli N, Jaafari MR, Daneshamouz S. (2006). Enhancement of follicular delivery of finasteride by liposomes and niosomes 1. *In vitro* permeation and *in vivo* deposition studies using hamster flank and ear models. Int J Pharm, 323:1–10.
76. Namdeo A, Jain NK. (1999). Niosomal delivery of 5-fluorouracil. J Microencapsul, 16:731–740.
77. Perrett S, Golding M, Williams WP. (1991). A simple method for the preparation of liposomes for pharmaceutical applications: characterization of the liposomes. J Pharm Pharmacol, 43:154–161.
78. Hal DAV, Bouwstra JA, Rensen AV, Jeremiasse E, Vringer TD, Junginger HE. (1996). Preparation and characterization of non-ionic surfactant vesicles. J Colloid Interface Sci, 178:263–273.
79. Carafa M, Santucci E, Alhaique F, Coviello T, Murtas E, Riccieri FM, Lucania G, Torrisi MR. (1998). Preparation and properties of new unilamellar non-ionic/ionic surfactant vesicles. Int J Pharm, 160:51–59.

80. Fang JY, Hong CT, Chiu WT, Wang YY. (2001). Effect of liposomes and niosomes on skin permeation of enoxacin. Int J Pharm, 219:61–72.
81. Vora B, KhopadeAJ, Jain NK. (1998). Proniosome based transdermal delivery of levonorgestrel for effective contraception. J Control Release, 54:149–165.
82. Schiffelers RM, Metselaar JM, Fens MH, Janssen AP, Molema G, Storm G. (2005). Liposome-encapsulated prednisolone phosphate inhibits growth of established tumors in mice. Neoplasia, 7:118–127.
83. Barakat HS, Darwish IA, El-Khordagui LK, Khalafallah NM. (2009). Development of naftifine hydrochloride alcohol-free niosome gel. Drug Dev Ind Pharm, 35:631–637.
84. Ruckmani K, Jayakar B, Ghosal SK. (2000). Nonionic surfactant vesicles (niosomes) of cytarabine hydrochloride for effective treatment of leukemias: encapsulation, storage, and *in vitro* release. Drug Dev Ind Pharm, 26:217–222.
85. Weiner N, Williams N, Birch G, Ramachandran C, Shipman C Jr, Flynn G. (1989). Topical delivery of liposomally encapsulated interferon evaluated in a cutaneous herpes guinea pig model. Antimicrob Agents Chemother, 33:1217–1221.
86. Betageri GV, Parsons DL. (1992). Drug encapsulation and release from multilamellar and unilamellar liposomes. Int J Pharm, 81:235–241.
87. Rogerson A, Cummings J, Florence AT. (1987). Adriamycin-loaded niosomes: drug entrapment, stability and release. J Microencapsul, 4:321–328.
88. Cocera M, Lopez O, Coderch L, Parra JL, de la Maza A. (2003). Permeability investigations of phospholipids liposomes by adding cholesterol. Colloid Surfaces A Physicochem Eng Aspects, 221:9–17.
89. Hofland HE, Bouwstra JA, Verhoef JC, Buckton G, Chowdry BZ, Ponec M et al. (1992). Safety aspects of non-ionic surfactant vesicles: a toxicity study related to the physicochemical characteristics of non-ionic surfactants. J Pharm Pharmacol, 44:287–294.
90. Wasan KM, Morton RE, Rosenblum MG, Lopez-Berestein G. (1994). Decreased toxicity of liposomal amphotericin B due to association of amphotericin B with high-density lipoproteins: role of lipid transfer protein. J Pharm Sci, 83:1006–1010.
91. Lee JW, Amantea MA, Francis PA, Navarro EE, Bacher J, Pizzo PA et al. (1994). Pharmacokinetics and safety of a unilamellar liposomal formulation of amphotericin B (AmBisome) in rabbits. Antimicrob Agents Chemother, 38:713–718.
92. Dupont B. (2002). Overview of the lipid formulations of amphotericin B. J Antimicrob Chemother, 49 Suppl 1:31–36.
93. Kirsh R, Poste G. (1986). Liposome targeting to macrophages: opportunities for treatment of infectious diseases. Adv Exp Med Biol, 202:171–184.