Optimizing Niosome Formulations for Enhanced Cellular Applications: A Comparative Case Study with L-α-lecithin Liposomes

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Abstract

Niosomes, emerging as nonionic surfactant-derived amphiphilic nanoparticles, hold substantial promise in the realm of biomedical research. This study addresses the need for a comprehensive exploration of niosome production optimization for biological applications, while also establishing meaningful comparisons with the well-established liposomal counterparts. Beyond conventional stability assessments, our motivation centers on discerning not only critical niosome process parameters but also on devising cost-effective, scalable alternatives to liposomes through comparative studies of liposomes and niosomes, rather than solely emphasizing niosomal stability advantages.

The primary objective of this study is to formulate and characterize a diverse array of niosomal nanoparticles, with a prime focus on their process-related parameters, physicochemical characteristics, cellular uptake, and toxicity performances. To establish the niosomes as their research twins of liposomes, the gap in the research field is picked as the starting point. The study is designed with stringent criteria based on the limitations of vasculature-tissue barriers. The proposed encompassing size (100-200 nm), polydispersity below 0.5, and zeta potential within the range of -10 to 10 mV are set for this purpose. These criteria serve as the initial screening parameters, streamlining the selection of niosome formulations with the potential to overcome the barriers. Through meticulous physicochemical characterization, we synthesized 10 optimized formulations aligned with the targeted size, polydispersity, and zeta potential ranges. In this physicochemical critical process parameter screening, short and long-term stability, shelf-life aggregation profiles, and the reproducibility of formulations were also assessed to confidently report the potential niosomal formulations for further drug delivery purposes. The statistical evaluations and analytical screening of process parameters obtained from the DoE interface indicated that most formulations maintain their critical criteria for at least 21 days, with three formulations remaining stable for 35 days. Reproducibility tests further validate the consistency of eight out of ten formulations regarding size, polydispersity, and surface charge. The F-score confirms high similarity between predicted and observed physicochemical properties (F-score = 0.83) for reproducibility tests.

Concurrently, we explore the pivotal process parameters governing niosome preparation and their consequential impact on physicochemical attributes. Further, physiochemically selected niosomal carriers are simultaneously exposed to cellular applications with L-α-lecithin liposomes including cellular toxicity and cellular uptake. In cellular toxicity, the selected niosomes from physicochemical screening were exposed to two different cancerous cell lines belonging to glioblastoma multiform (U-87 MG) and lymphoblast-like cell line (NFS-60). The cellular uptake profiles in U-87 MG and simultaneous comparison with liposomes revealed non-toxicity across all formulations and promising cellular uptake performance in four formulations, either similar to or better than liposomes.

Overall, this study holds potential implications of niosomes for advancing reliable drug delivery strategies, enhancing treatment efficacy, and ensuring safety in various therapeutic applications. Besides, demonstrating the scientific records of physiochemically controlled niosomes' similarity to a type of liposomes in cellular interactions and scalable production will ultimately expand their applications in the field of biomedical research.

1. Introduction

Niosomes are one of the promising pseudo-lipid nanostructures for the delivery of natural agents and drugs, which have gained acceptance as structural analogs of liposomes in the development of drug delivery strategies. Niosomes have become particularly popular for drug delivery in recent years due to their amphiphilic, biocompatible, biodegradable, and nontoxic properties. Unlike liposomes, niosomes have a vesicle construct that contains nonionic surfactants and forms a bilayer that provides better bioavailability by increasing residence time and decreasing renal clearance.[(1)–(4)] Niosomes are useful not only for drug delivery but also for targeted drug transport mechanisms such as antigens and small molecules.[(5)] Due to the use of nonionic surfactants, they are a cost-effective alternative; their longer chemical stability also makes them a promising candidate for drug delivery applications.[(1),(4),(6),(7)] They can encapsulate the drug into vesicles that improve drug bioavailability, therapeutic efficiency, and penetration through the tissue, release the drug in a sustained and controlled manner, and can be targeted to the desired site by adjusting the composition, which helps to reduce side effects.[(8)]

While numerous studies have highlighted the advantages of lipid nanoparticles as intelligent drug carriers with both active and passive targeting capabilities,[(9)–(11)] there is a parallel development of niosomal nanoparticles that are designed to be more cost-effective, exhibit improved encapsulation yields, and result in stable and reproducible nanocarrier formulations.[(12)] Nevertheless, what remains conspicuously absent is a simultaneous and comprehensive comparison with liposomes, particularly with regard to their performance in cellular applications. This comparison is crucial for establishing niosomes as a genuinely cost-effective alternative to liposomes. In addition, the influence of surfactants on the physical properties of niosomal nanoparticles has been explored as an enhancer of stability when compared to lipid nanoparticles. However, it is worth noting that the majority of studies have predominantly focused on drug-loaded lipid or pseudo lipid nanoparticles, particularly concerning *in vitro* testing and drug-release profiling. Unfortunately, relatively little attention has been devoted to investigating the properties and characteristics of niosomal nanoparticle formulations and assessing their differences and similarities in cellular performance when compared to liposomes. Such investigations are vital for their effective utilization in drug delivery applications.

The critical process attributes for reproducing niosomal nanoparticles and achieving appropriate cellular interactions with them are main considerations, specially from the drug delivery framework.[(2),(13)–(17)] The design of excipients for drug development requires compliance with several commitments, including optimization of the nanostructure formulation with respect to factors such as penetration, minimum effective concentration, minimum toxic concentration, bioavailability at the site of action, frequency and route of administration, and physicochemical state.[(18)] Because of obligations, the carrier formulation and its optimization are as important as the drug itself.[(18)] To achieve the optimal drug carrier formulation, it is critical to characterize the parameters related to nanoparticle geometry[(19)], physicochemical properties[(6),(7),(20),(21)], cellular toxicity[(20),(22)], cellular uptake[(23),(24)], and species-specific abundances[(25),(26)].

Few studies have used formulation-dependent optimization strategies, yet, niosomal formulations can be quite critical in biological applications, as demonstrated by the example of surfactant composition and surface charge of niosomes, which have a tremendous impact on the oral adsorption of repaglinide.[(27)] On the other hand, the influence of surfactant type and production parameters such as concentration, sonication time, temperature, and surfactant/cholesterol ratio on drug delivery has not been fully elucidated.[(27),(28)] There is ample evidence in the

literature that niosomes are an alternative to liposomes in terms of efficacy, safety, and stability, but the lack of reliable and reproducible niosomal formulations remains still as a limitation for transiting of these literature findings into the clinic.[5] Therefore, it is necessary to conduct further characterization studies of niosomal formulations to optimize the process parameters for their reliable and reproducible production.

Here we emphasize the importance of preliminary analytical characterization of drug carriers, niosomes, and propose several niosome formulation alternatives with critical process parameter evaluation. Overall, preliminary physicochemical characterizations, cellular toxicity, cellular uptake, and formulation-specific frequencies to address carrier-mediated critical parameters and simultaneous comparison with liposome nanostructures from the same analytical perspective provide evidence to the literature on the compatibility of niosomes, with the goal of more cost-effective production approach than liposomes for drug delivery, not only for research-based studies but also for pharmaceutical industry. *Figure 1* shows the general outline of the study and clarifies the perspective of the study.

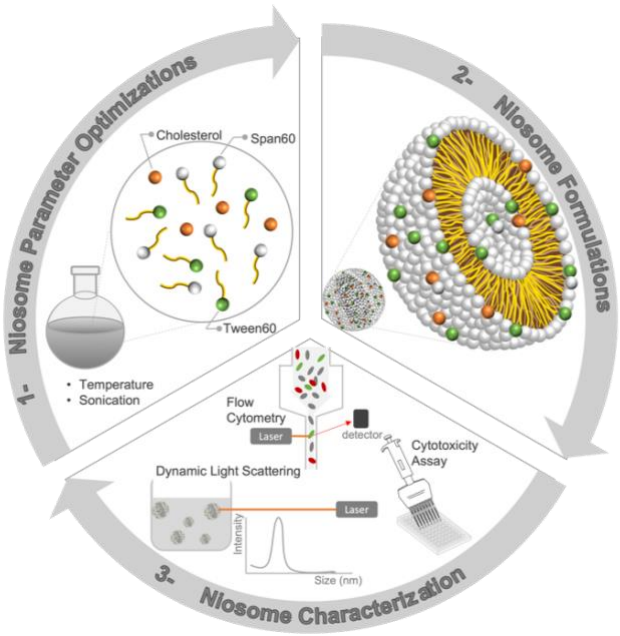


Figure 1. Overall study perspective for determining quality attributes and critical process parameters of niosomes used in drug delivery system.

2. Materials and Methods

Materials

Span 60 (Sigma Aldrich, Germany), Tween 80 (Sigma Aldrich, Germany), fluorescein isothiocyanate (FITC) (Sigma Aldrich, Germany), L-α-lecithin, and Cholesterol (Chol) (Sigma Aldrich, Germany) were purchased from Sigma-Aldrich Chemical Co. Chloroform was purchased from Merck (Germany). 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck (Germany). The U-87 cell line was a kind gift from Esendagli Group at Hacettepe University (Turkey). The NFS-60 cell line was a gift from ILKOGEN Biotech. All organic solvents were analytical grade and deionized distilled water was obtained from Merck Millipore with a fixed 18.2-ohm conductivity.

Preparation of niosomes

Various niosome formulations (*Table 1*) containing different types of surfactants and molar ratios were prepared using the thin-layer hydration technique.[29] The appropriate amount of cholesterol and surfactants were dissolved in a mixture of ethanol and chloroform (0.1:1 v/v) in a round bottom flask with a final volume of 50 mL. The formulations are classified as multiple surfactant type formulations (MSTF) and single surfactant type formulations (SSTF). 3 different molar ratios for MSTF and 2 types of surfactants for SSTF in a molar ratio of 1:1 with cholesterol were established as preliminary niosome characterizations. A total of five different formulations were formed and designated as Formulation A, Formulation B, Formulation C, Formulation D and Formulation E (Table 1). Four different final concentrations were prepared, and two different sonication times were applied. The organic phase was removed at 60°C under vacuum using a rotary evaporator (Heidolph, Germany). The vacuum time was set at 1 hour for all formulations, and the dried thin film was hydrated with 10 mL of phosphate buffer saline (PBS) (Sigma-Aldrich Co., Germany) at 60°C by gently shaking the round bottom flask until all the dried film was dissolved. Each prepared formulation was treated in a sonication bath (ISOLAB, Turkey) for 45 and 90 minutes, respectively.

Table 1. Preliminary screened formulation A, B, C, D, and E ingredient and condition list

Formulations	Span 60 (%)	Cholesterol (%)	Tween 80 (%)	Formulation Type	Final Concentration (mM)	Sonication Time (min)
A	25	50	25	MSTF	0.1 mM; 0.2 mM; 0.3 mM, 0.42 mM	45 mins & 90 mins
B	50	50	-	SSTF	0.1 mM; 0.2 mM; 0.3 mM, 0.42 mM	45 mins & 90 mins
C	-	50	50	SSTF	0.1 mM; 0.2 mM; 0.3 mM, 0.42 mM	45 mins & 90 mins
D	30	40	30	MSTF	0.1 mM; 0.2 mM; 0.3 mM, 0.42 mM	45 mins & 90 mins
E	35	30	35	MSTF	0.1 mM; 0.2 mM; 0.3 mM, 0.42 mM	45 mins & 90 mins

Preparation of niosomal nanoparticles

In the first phase, the synthesis of nanoparticles is adjusted to the desired size range of 100-200 nm and homogeneity in the range of 0.2-0.5.[30] In order to achieve the desired physicochemical properties of the niosomal nanoparticles, the "thin-film layer" method was used.[13] *Figure 2* describes the method in 6 different steps of the preparation of niosomal nanoparticles.

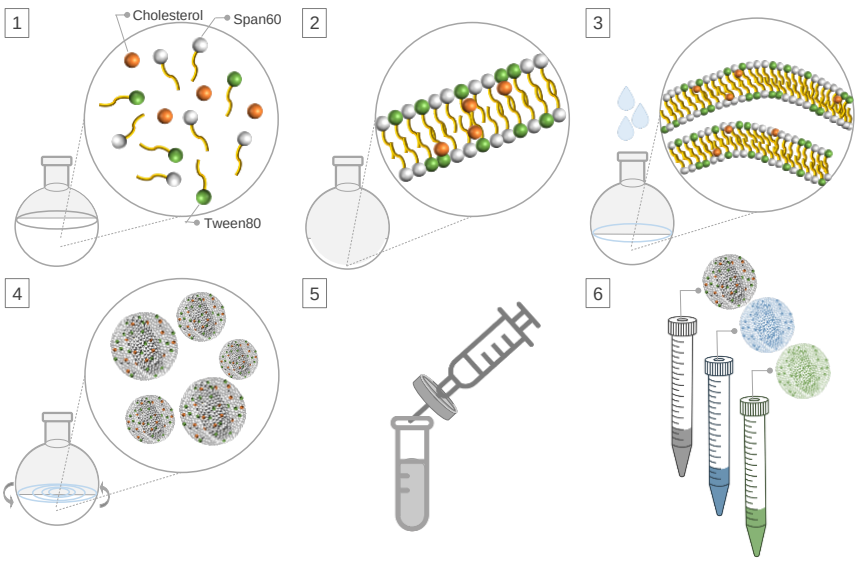


Figure 2. Thin-film layer method for preparation of niosomes: 1) Surfactant and cholesterol mixture, 2) Evaporation of organic solvent, 3) Hydration, 4) Sonication, 5) Filtration, and 6) Collection of final niosomal nanoparticles.

Step 1: In part 1, surfactants and cholesterol constructs are added to round-bottom open-ended bottles. For this, 100 mL round glass bottles can be used, but larger or smaller volumes can be used later request. The amount of cholesterol and surfactants will be explained in the next step, namely method optimization. These two substances will be dissolved in chloroform organic and volatile solvents.[31]

Step 2: Section 2 shows the rotary evaporator stage. Chloroform, an organic volatile solvent, dissolves cholesterol and surfactants and a homogeneous liquid is obtained. Then, using a rotary evaporator, the chloroform is volatilized with the help of vacuum and temperature. The temperature applied here is the "glass transition temperature" known as the glass transition temperature, and it has been determined as 60°C.[31],[32] The vacuum value was determined by selecting 470 psi in the range of 470-520, which is the psi value at which chloroform is expected to evaporate. Here, the average flight time is determined to be 30 minutes. After the rotary evaporator, a thin layer is deposited at the bottom of the round bottom flasks with the effect of temperature and vacuum. This thin layer has a whitish texture and is completely adhered to the bottom of the bottle.

Step 3: The stage shown in section 3 is the hydration stage. At this stage, the layer accumulated at the bottom of the round bottom bottles is subjected to hydration with the help of water or PBS. PBS was used at this stage. It is also important how much PBS is added and hydrated. The process was carried out by adding an average of 10 mL of PBS to each bottle.

Step 4: The step shown in section 4 is the sonication step. The hydrated layer is dissolved in an ultrasonic mixer (ISOLAB Co.), preheated to 60°C and became milky white. These are synthesized pseudo-lipid nanoparticles. At this stage, the milky white liquid is left for prolonged sonication. Sonication time and sonication temperature are important.

Step 5: Since the sonication time and temperature are directly related to size and homogeneity, optimization at this stage is important. Hydrated solution is filtered by 0.2 µm sterile filters (ISOLAB Co, Germany.).

Step 6: Finished and filtered products of various niosomal formulations are diluted 1:10 ratio, transferred to 15 mL falcon tubes (ISOLAB Co.). Synthesized liquid pseudo-lipid nanoparticles are subjected to homogeneity, size, and surface charge analysis with Dynamic Light Scattering (DLS) - Malvern device.

Preparation of liposomal nanoparticles

Liposomes were prepared by thin film hydration method.[33] L-α-lecithin (3.3 mg/mL) and cholesterol (0.8 mg/mL) were dissolved in chloroform. The lipid mixture in chloroform solution was placed in a round bottom flask and the chloroform was evaporated using rotary evaporator (150 rpm, 55°C, 332 mbar) to form a lipid film on the sides of the round bottom flask. The resulting dry lipid film was hydrated with 3 mL of phosphate buffered saline (pH 7.4). For extrusion, the resulting lipid vesicles were passed through polycarbonate membranes of 0.6 μm, 0.4 μm and 0.2 μm pore sizes, respectively. Liposomes were then placed in a dialysis bag (3500 Da MWCO, regenerated cellulose membrane, Sigma Aldrich, Germany) and dialyzed against phosphate buffered saline (pH 7.4) for 16 hours. Size, polydispersity, and zeta potential measurement data for liposomes were given in the *Supplementary Table 7*.

DLS Measurements

Physicochemical characterization was obtained by Dynamic-Light Scattering (DLS) (Malvern PanAnalytical, United Kingdom) with fixed and optimized experimental procedure. Briefly, each formulation was prepared as triplicates and 13 iterations were arranged for each run. 1:10 diluted PBS at pH:7.4 was assigned as blank before each triplicate to zeroize the system. Viscosity of niosomes was set as 1.48 ± 0.01 (mPa.s).[34] To measure the size and homogeneity of formulations, polystyrene cuvettes (Malvern PanAnalytical, United Kingdom) were used. To measure zeta potential of each formulation, capillary zeta cell cuvettes (Malvern PanAnalytical, United Kingdom) were used.

Fluorescence labelling of niosomes

Niosomes and liposomes were labelled with fluorescein isothiocyanate (FITC; Sigma-Aldrich) by passive adsorption at 2-8°C overnight, following the previously published protocol.[35],[36] Each niosome formulation is tagged by 1:5000 FITC from 300 nM stock solution prepared in PBS at pH: 7.4 with two different mixing ratios (1:50 and 1:100 v/v) by thin-film hydration. Excessive FITC was removed by continuous sterile filtering. Encapsulation efficiency of FITC was calculated based on the former FITC-load absorbance and after the centrifugal FITC-load absorbance. The FITC concentration was calculated based on FITC-based standard curve demonstrated in *Supplementary Figure 6*. Each labelled formulation was physiochemically characterized after the labeling procedure by DLS with a dilution factor of 1:10 in PBS to determine whether its behavior changed in terms of size, charge, and polydispersity. The tagging of niosome-FITC was only generated for assessing the cellular uptake. Encapsulation efficiency of niosomes were performed with the 1:10 diluted niosomes. The niosomes were exposed to ultracentrifugation at the maximum speed to burst the niosomes and supernatants were collected. Released FITC absorbance was recorded as triplicates and calculated based on FITC standard curve, demonstrated in *Supplementary Figure 6*.

FTIR Analysis

FTIR analysis was performed to verify the presence of FITC in the niosomal nanoparticles and to ensure the labeling procedure. The samples were dried by freeze-drying in glass containers. The control groups consisted of

FITC only and nanoparticles only to compare whether the labeling was successful. The dried samples were analyzed by FTIR (ThermoFisher, USA) and analyzed using OMNIC® software. The statistical evaluations and the raw version of the data analyzed with the software are given in the *Supplementary Figure 8*.

Cell maintenance

Glioblastoma cell line U-87 and lymphoblast cell line NFS-60 were grown as adherent and suspension cells in Dulbecco's Modified Eagle Medium (DMEM) (Pan Biotech, Germany) containing 10% FBS + 1% Penicillin/Streptavidin and RPMI 1640 (Pan Biotech, Germany) + 10% M-CSF (Gibco), respectively. Cells were cultured in T75 culture flasks (ISOLAB Co, Germany.) at the same period (3-day culture interval) with a division ratio of 1:5.

Cell viability assay

All cell viability assays were performed in 96-well plates (ISOLAB Co.) by seeding 5000 cells/well in 100 µL to each well with 2 hours incubation at 37ºC. 9 serial dilutions with 1:10 dilution from 1000 pM to 10^{-5} pM. All dilutions were in starvation media with 1:2 volume ratio. Niosomal nanoparticles were incubated with the cells 24, 48, or 72 hours. Then, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was dissolved in PBS at pH: 7.4 with a final concentration of 0.5 mg/mL, applied 50 µL on each well of the plated, incubated 3 hours. Reading of the wells was performed by removing media and adding 200 µL desolubilization solution containing 10% SDS in DMF at pH:4.6. The results of cell viability assays were obtained using a microplate reader (BioRad, USA).

Flow cytometry assay

Cell lines were seeded in 24-well plates (ISOLAB Co.) at a seeding rate of 2 x 10^4 cells/well. Seeded cells are adapted overnight before the assay. FITC-tagged niosomes were diluted 1:5000 and added as 1:100 ratio of total volume of the well. Passive incubation was followed by the intervals of 2, 4, 6, 8, and 10 hours and collected separately with their duplicates, demonstrated in *Supplementary Figure 7*. Trypsin (Pan Biotech) was used to remove the cells for replacing medium by centrifuge at 2000 rpm, 5 mins with PBS and repeating this step continuously three times. Final solution of cell-uptake niosomes are dissolved in PBS containing 1% PFA. After optimization, total cellular uptake was performed after 6 hours of incubation. Cell uptake analysis of each formulation was tested using FACS (BD, USA) with FITC-tagged niosomes. All cell uptake analyzes, including the control group, were analyzed using FlowJo® software and structured by layout mode with histogram-based gating.

MODDE analysis

*P*reliminary formulation screening for investigation of final concentration, sonication time, surfactant type, and surfactant composition was evaluated by *MODDE*® Software. Design model was set based on target size range as 100 – 200 nm. Modeling was determined as multifactorial analysis and significance was set as alpha: 0.5. Contour mapping mode and screening module were selected to obtain optimized formulations and 10 different optimization formulations were obtained. *Table 2* indicates *MODDE*® Software output evaluated in logarithmic threshold.

Statistical analysis

Prism (License number: GPWF-045319-RIE-6746 to Sabanci University, Version 5.01) was utilized for experimental statistical evaluations. One-way, two-way, three-way ANOVA analysis with one sample t-test confirmations were used for data analysis. Cell viability assay was analyzed by Graph Prism® log-response sigmoidal analysis mode.

3. Results & Discussion

Physicochemical Characterization

Although many studies have focused on optimizing the process for drug-loaded niosome formulations in terms of entrapment efficacy (EE%) and release assays[(17),(37)–(39)], few studies have been conducted to understand the physicochemical properties of niosomes with or without drug load. For instance, Taymouri et al. investigated the effect of different surfactants on the physical properties of carvedilol nano-niosomes using 40% and 50% of the cholesterol with 25% or 30% Span/Tween 60 and concluded that the Span/Tween 60-containing formulation had the highest stability due to the high transition temperature and solid-state properties of these surfactants.[(12)] Yeo et al. investigated the effect of hydration parameters and co-surfactants on methylene blue-loaded niosomes, using Trizma® buffer (pH 7.4) for hydration, and three different hydration volumes (mL) and two different hydration times (min) were tested, with a fixed but unreported sonication time, at two different storage temperatures for four weeks.[(16)] The size ranges (nm) and polydispersity (PDI) were recorded before and after the sonication time by changing the final lipid content.[(16)] However, the impact of overall process parameters such as surfactant concentration, cholesterol concentration, comparison of each surfactant formulation and their effects on physicochemical properties were not reported. Therefore, in this study, we aimed to fill this knowledge gap and reveal the effects of surfactant type and composition ratio on niosomal nanoparticles.

We started with a screening of the production parameters of niosomes and their mutual synergistic effects. We used the thin-layer film hydration method for niosome production, with each step contributing to the variables and constants of physicochemical characterization. These process parameters included surfactant type, constituent molar ratios, number of surfactants, final suspension concentration (mM), sonication time (min), transition temperature (ºC), rotation speed (rpm), organic solvent type, final suspension volume (mL), hydration buffer, and hydration volume (mL). However, the effects of many of these process parameters on the physicochemical properties of niosomes, such as size, polydispersity, and zeta potential, were unknown or had not been published before.

To perform an initial screening of the process parameters, we conducted an experiment with niosome production parameters as listed in *Table 1*. We divided the formulations into single-surfactant type formulations (SSTF) and multi-surfactant type formulations (MSTF), each with four different concentrations (mM) and two different sonication times (min). The temperature was 60ºC, the rotation speed for the hydration step was 150 rpm, the organic solvent was chloroform, hydration buffer was phosphate buffer saline (PBS), and the hydration volume was 10 mL throughout the study. Our main objective was to understand the effects of concentration (mM), sonication time (min), and surfactant types (MSTF vs. SSTF) on the physicochemical properties of niosomes such as size (nm), PDI, and zeta potential (mV). *Figure 3* shows the overall size (nm) distribution as a function of all

process variables across SSTF and MSTF of niosomes, while *Supplementary Figure 2* indicates size distribution across five different formulations at two different sonication times (45 mins vs. 90 mins). *Supplementary Figure 1* shows the polydispersity and zeta potential (mV) distribution profiles at different concentrations (0.1 mM, 0.2 mM, 0.3 mM, and 0.42 mM) for all formulations. To consider concentration variable (controlled) to size variable (uncontrolled), two-way ANOVA statistical consideration is applied. By that, the effect of concentration on size was aimed to be determined. To consider the separate investigation for sonication time on the concentration variable, while sonication time was already having two different set points, unpaired t-test was applied between size and sonication time to investigate the correlation between. The effect of sonication time on the size of five different formulations (as shown in *Supplementary Table 1*). *Supplementary Table 4A* and *4B* provide further details on two-way ANOVA for the PDI and zeta potential of the preliminary formulation screening of niosomes, in addition to sonication time versus size consideration.

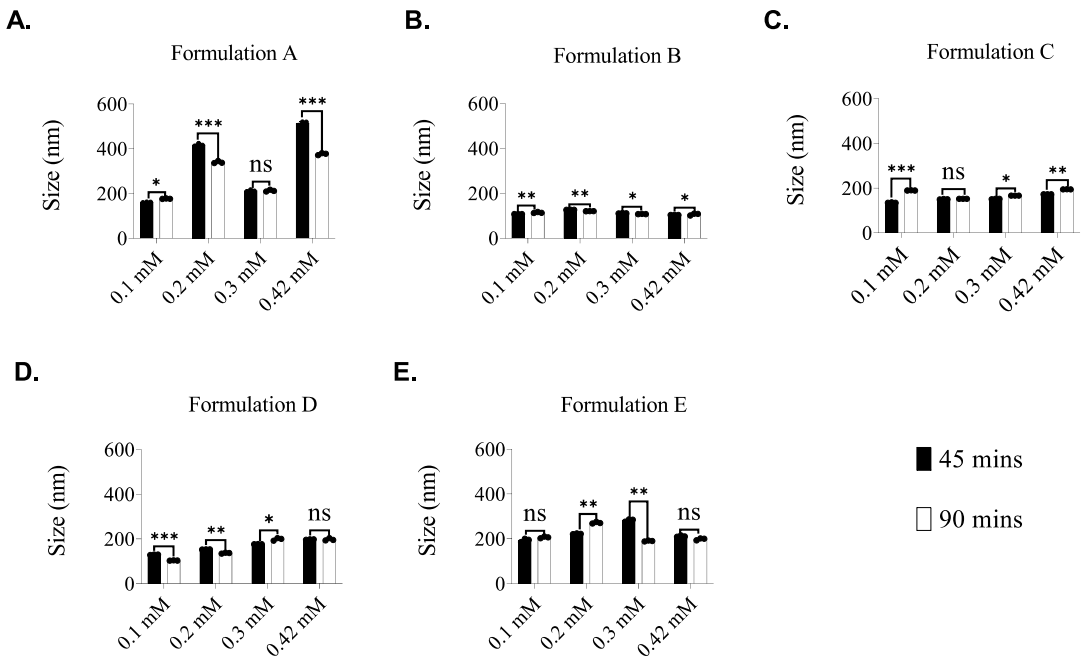


Figure 3. Sonication effect (45 mins vs. 90 mins) profile in different concentrations (0.1 mM, 0.2 mM, 0.3 mM, and 0.42 mM) for all formulations. Two-way ANOVA analysis is applied to all preliminary formulations to identify concentration (mM) and sonication time (min) on size (nm). ns: p-value > 0.05; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001, ****: p-value ≤ 0.0001

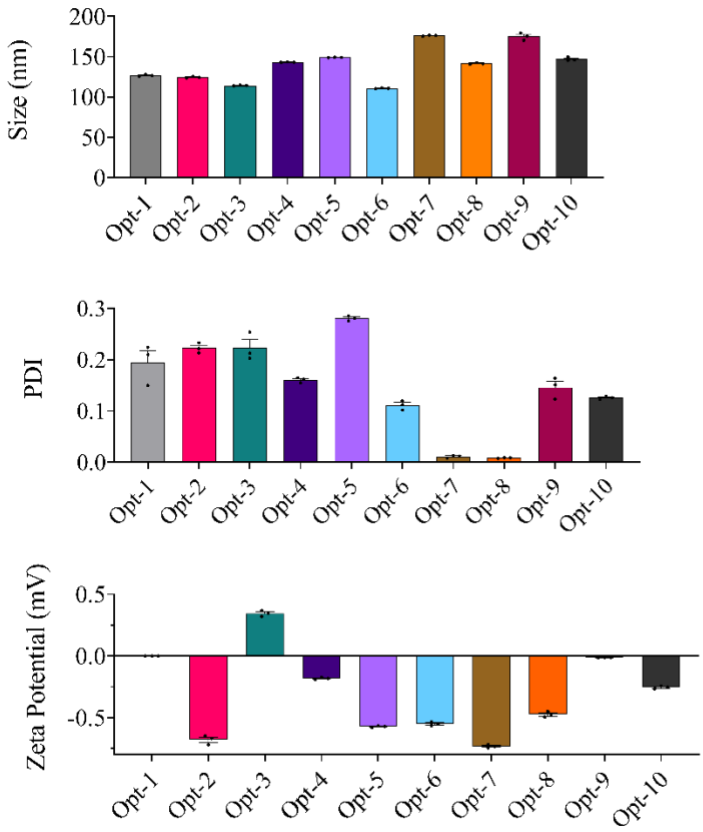
The correlation matrix shown in *Table 2* combines two-way ANOVA and multiple t-tests. The optimal process conditions for niosome production were determined by screening three parameters: 1) ingredients, 2) final concentration, and 3) sonication time. The first result showed a synergistic effect of final concentration (mM) and sonication time (min), which had the highest statistical significance (****: p-value ≤ 0.0001). Another important finding was that the cholesterol-Span 60 (SSTF) combination was less significant (*: p-value ≤ 0.05) compared to the cholesterol-Tween 80 (SSTF) pairs (***: p-value ≤ 0.001). When both Span 60 and Tween 80 were used together (MSTF), the significance on the niosome production increased to the lowest p-value (****: p-value ≤ 0.0001). Interestingly, sonication time (min) or final concentration (mM) alone did not significantly affect the

production process. However, when considered together, MSTF was more affected by sonication time (min) and concentration (mM) than SSTF, and both were found to have a synergistic effect.

Table 2. Correlation matrix of ingredients, final concentration (mM), and sonication time (min). (NS: p-value > 0.05; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001, ****: p-value ≤ 0.0001.)

X	Cholesterol (%)	Span 60 (%)	Tween 80 (%)	Final Concentration (mM)	Sonication time (min)
Cholesterol (%)	-	*	***	NS	NS
Span 60 (%)	*	-	****	NS	NS
Tween 80 (%)	***	****	-	NS	NS
Final Concentration (mM)	NS	NS	NS	-	NS
Sonication time (min)	NS	NS	NS	NS	-

The data obtained were statistically analyzed and further formulations were determined using the DoE program MODDE®. The optimized conditions were evaluated by a logarithmic order with the evaluation of the target size between 100-200 nm. PDI and zeta potential (mV) were eliminated for the targeting step. MODDE® proposed 10 different optimized experimental conditions, which are shown in *Table 3.* By applying the proposed conditions by MODDE, we obtained niosomal nanoparticles, and their size distributions (nm) represented in a single column in *Table 3* and their detailed size (nm), PDI, and zeta potential were shown in *Figure 4*. For the evaluation of these three parameters, the size (nm) was set at 100–200 nm to ensure the nanoparticles entry into cellular and biological barriers.[(40),(41)] To obtain homogeneously dispersed niosomes, the critical PDI range was kept between 0.2-0.5.[(42)] Since no surface charge agents were added, the expected of zeta potential (mV) was nearly neutral. However, to set the critical range for zeta potential (mV), biologically acceptable range kept as -10–10 mV.[(43)] Based on all preliminary set-up target ranges in size (nm), PDI, and zeta potential (mV), all obtained data were considered to be within the critical range. The predicted formulations were also compared with experimentally obtained data by applying the similarity test (F-score: 0.83). According to the obtained average size (nm), the overall size range of 10 optimized formulations was between 120 and 180 nm, the PDI range was between 0.1 and 0.3, and zeta potential (mV) range was between -0.5 mV and 0.5 mV.



Formulation	SD	MEAN
Opt -1	0,784	126,67
Opt-2	0,634	124,43
Opt-3	0,454	114,21
Opt-4	0,286	143, 23
Opt-5	0,249	149,03
Opt-6	0,368	110,76
Opt-7	0,555	176, 16
Opt-8	0,713	141,53
Opt-9	3,741	175,16
Opt-10	1,913	147,1

Figure 4. 10 different optimized niosome formulation physicochemical profiles **A.** Size distribution (nm) (Ranging from 100 nm to 200 nm) **B.** Polydispersity (PDI) (Ranging from 0.05 to 0.3) **C.** Zeta Potential (mV). (Ranging from -0.5 mV to 0.5 mV)

Table 3. DoE optimized formulation, ingredient, and condition list

Molar Ratio (%) (CHO: S60: T80)	Predicted Size (nm)	Predicted Concentration (mM)	Predicted sonication time (min)	Obtained Size (nm)
34.5: 43.5: 22	100.6	0.228	63	127.7 ± 7.11
41: 2: 57	99	0.126	47	125.4 ± 2.13
40: 3: 57	92.5	0.1	45	114.7 ± 14.14
49: 49: 2	97	0.125	86	142.9 ± 11.32
49: 49: 2	92	0.1	83	149.3 ± 12.11
48: 4: 48	99.8	0.11	50	110.3 ± 9.22
49: 48: 3	99.5	0.13	89	176.7 ± 11.12
50: 50: 0	84.4	0.1	90	141.3 ± 15.48
50: 50: 0	92	0.1	45	137.3 ± 9.23
50: 50: 0	97	0.2	90	145.6 ± 5.34

Stability Test

10 different optimized niosome formulations were characterized and found to be in the critical range of size (nm), PDI, and zeta potential (mV). Niosomes are considered to be more stable drug carriers than liposomes, but accurate stability profiles of niosomes have not been established beyond 4 weeks.[12] On the other hand, although size (nm) was followed up to 4 weeks, their PDI and zeta potential (mV) fluctuations by time were not reported. Therefore, a 92-day stability test was performed while storing the samples at +4ºC. Each formulation and the variations in its size (nm), PDI, and zeta potential (mV) were compared by applying one-way ANOVA. *Figure 5* shows the change in size (nm) in 92 days for each formulation. Formulations were named according to *Table 3* as the order of columns from Opt-1 to Opt-10. *Supplementary Figure 2* demonstrates PDI and zeta potential (mV) profiles in 92 days for each optimization. The most stable niosome formulations in terms of size (nm) were defined based on slight changes and no aggregation profiles in their peak iterations. The most stable PDI and zeta potential (mV) profiles of niosome formulations were determined based on sudden jumps in PDI and zeta potential (mV). Slight changes were also acceptable decision criteria for PDI and zeta potential (mV). Detailed statistical analysis is presented in *Supplementary Table 5A* and *5B.*

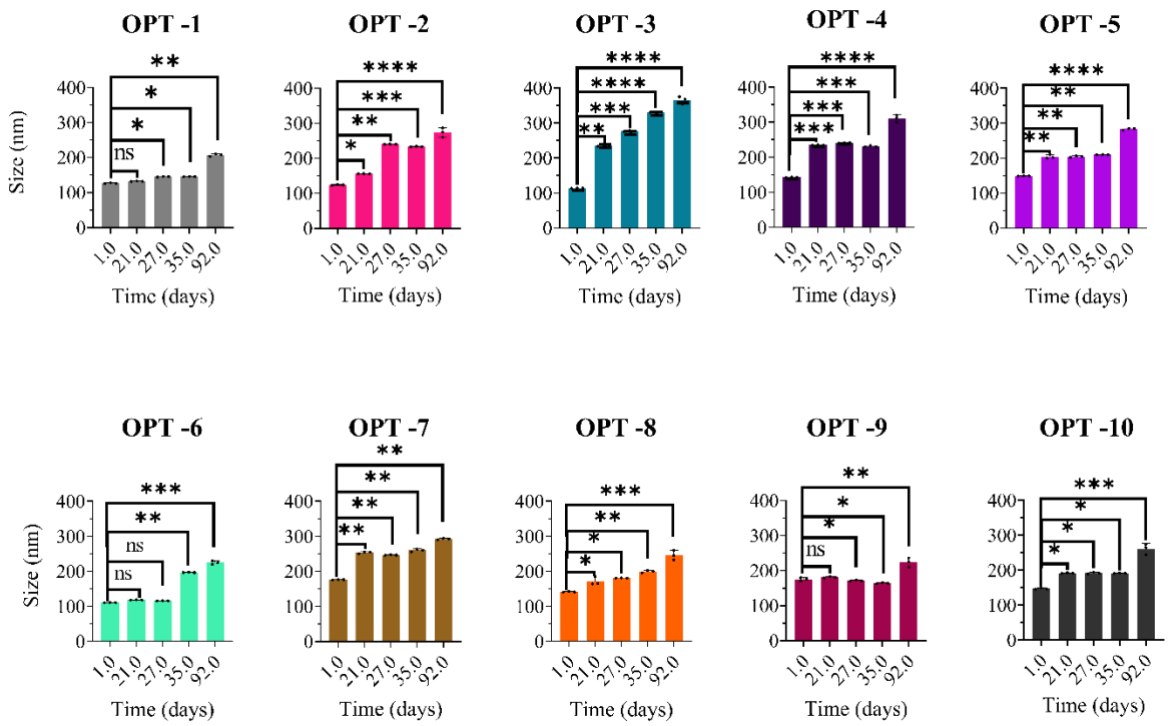


Figure 5. 92 days shelf-life profiling of optimized 10 formulations. One-way ANOVA analysis is applied to all niosome formulations to determine overall 92 days shifting profile for A. Polydispersity (PDI), B. Zeta Potential (mV). One sample t-test is applied to each formulation and analyzed for the shift between 1-21 days, 1-27 days, 1-35 days, and 1-92 days in *Supplementary Table 5A* and *5B.* (ns: p-value > 0.05; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001, ****: p-value ≤ 0.0001.)

Supplementary Table 5A and *5B* demonstrated the detailed statistical significance evaluation in their stability profiles for PDI and zeta potential (mV). In the size stability profiles, Opt-1, Opt-6, Opt-8, Opt-9, and Opt-10 showed slight changes in 92 days and were also in the critical size range, which was set at 100–200 (nm). The PDI stability profiles demonstrated that Opt-4, Opt-5, Opt-9, and Opt-10 had slight changes in 92 days. Except for Opt-1, Opt-2, and Opt-3, the PDI of all optimized niosome formulations was in the critical range of 0.2-0.5. The zeta potential (mV) stability profiles were in the critical range of -10 to 10 mV for all niosome formulations. However, the most stable zeta potential (mV) profiles were selected as Opt-5, Opt-6, Opt-9, and Opt-10 to avoid sudden changes. Interestingly, Opt-9 was found to have a gradual increase in electronegativity over 92 days among all other optimization formulations, which was still in the range of -10 to 10 mV. Stability tests showed that almost 5 niosome formulations were in the critical size range (nm), almost 7 niosome formulations were in the critical PDI range, and all niosome formulations were in the critical zeta potential (mV) range in 92 days.

Reproducibility Test

To determine the reproducibility of the niosome formulations, three experiments were performed at three different days under the same conditions as in *Table 3*. Comparison was made using F-score (Sorensen-Dice coefficient). The F-score test indicates the similarity of each run with respect to the different intervals between days and gave a value of 0.62. The most reproducible data among 10 formulations were obtained with Opt-10, one of the most stable optimized conditions in the size range from 100 nm to 200 nm. The reproducibility test was necessary to use this nanocarrier formulations in drug delivery with greater accuracy. Additionally, the reproducibility test, followed by a 92-day stability test, brought the novel results to the literature where no record of them was found before. *Figure 6* demonstrated the reproducibility profile for the size (nm) and *Supplementary Figure 3* demonstrated the reproducibility profile for the PDI and zeta potential (mV). Based on the reproducibility profiles for the size (nm) of 10 niosome formulations, all formulations except Opt-5 and Opt-7 can be said to be reproducible. Although significant change was observed in some optimizations except Opt-5 and Opt-7, all niosome formulations were found to be reproducible due to the non-excessive profile in their critical ranges of size (nm), surface charge (mV), and polydispersity. Detail statistical evaluations can be found in *Supplementary Table 6.*

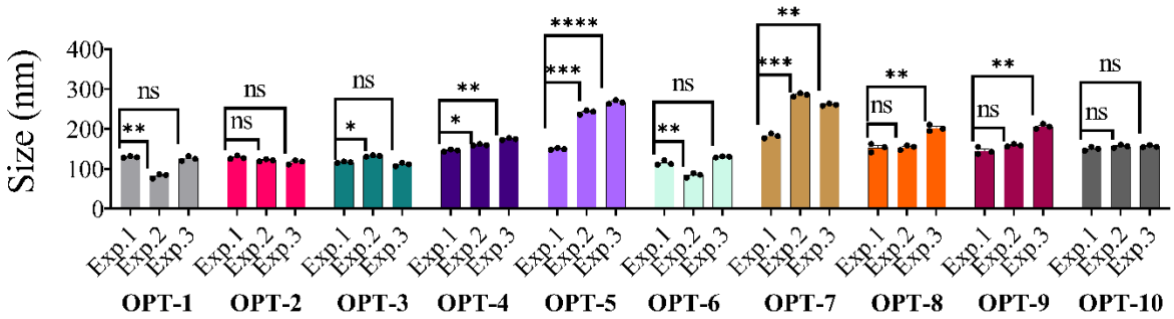


Figure 6. Intraday experiments of 10 optimized formulations demonstrating reproducibility performance of each in size (nm). PDI and Zeta Potential profiles are demonstrated in *Supplementary Figure 3*. Unpaired t-test ANOVA statistical analysis is performed to compare and determine three inter-day experiment group in same experiment conditions. ns: p-value > 0.05; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001, ****: p-value ≤ 0.0001.

Cellular Toxicity

Physicochemical characterization of niosome formulations, stability profiles and reproducibility tests were performed, and promising formulations were recorded. To test their cellular toxicity, MTT assay was performed two different cell sources: U-87 and NFS-60. *Supplementary Figure 4* shows the cellular viability profiles of 10 different niosome formulations and liposomes of the glioblastoma cancer cell line U-87. We screened the cellular viability profiles of these niosomal formulations after 48 days of their synthesis to determine whether the range of cellular viability had gradually shifted, as shown in *Figure 7* for U-87. In addition, the leukemia cell line, NFS-60 was tested with the same niosome formulations and liposomes (see *Supplementary Figure 5).* Both of these different cell lines with the same niosome and liposome formulations showed no cellular toxicity and had similar cellular viability profiles. On day 1, cellular viability ranged from 80% to 100%. On day 24 and day 48, cellular viability ranged from 75% to 100%. The results also confirmed the stability test of the niosome formulations, in almost all niosome formulations were stable for at least 2 months. In order to make final decisions for further selection of optimal niosomal formulations, their cellular uptake profiles needed to be investigated.

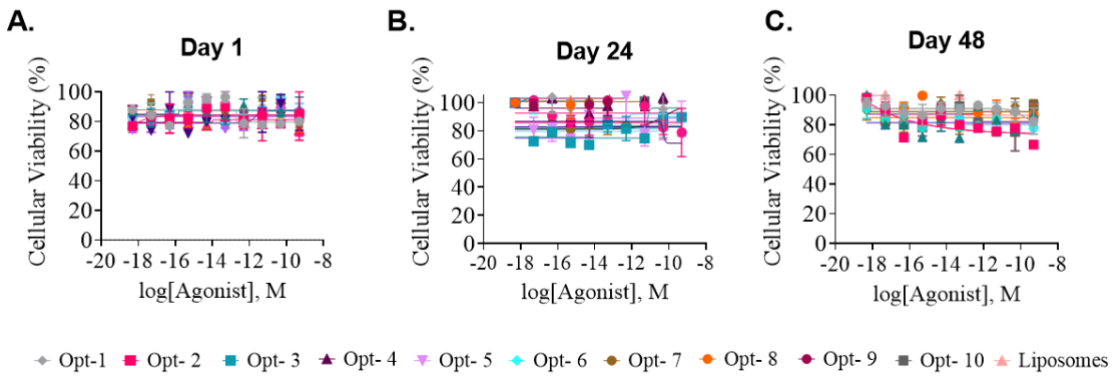


Figure 7. 48 days cellular viability MTT assay for all niosome optimizations and liposome comparison performed in U-87 cell line. A. Day 1 cell viability (%) in the range of 80-100. B. Day 24 cell viability (%) slow shift to the range of 75-100. C. Day 48 cell viability (%) slow shift to the range of 75-100. 5000 cells/wells are used, and 9 serial dilutions are applied. Sigmoidal log-response concentration (M) indicates the x-axis.

FITC-Labelled Niosomes

FITC labelling of niosomes were based on the total volume of niosomal formulations before the production process. The overall, based on the desired last concentration, FITC existence was set to 0.05% of the total volume from the stock solution of 300 nM. The percentage of FITC and stock solution concentration are set, and encapsulation efficiency was calculated based on sole standard curve of FITC. Standard curve of FITC and the linear curve equation was presented in *Supplementary Figure 6.* Based on the equation, the known concentration of FITC before load was measured triple. After encapsulation of FITC, high-speed centrifugal release (12,000 rpm, 20 mins) was used, and supernatant absorbance was recorded triple to calculate the entrapment efficiency. Results demonstrated that the encapsulation efficiency (%) for FITC-tagged niosomes were 89,2 % ± 1,5.

FITC Labelled niosomes are produced to be analyzed in the cellular uptake analysis performed by flow cytometry. The analysis conditions were set based on the sole population FITC-A mean intensity which was asset for the starting gate. The sole population was supposed to not have FITC-A count. However, FITC autofluorescence positive feed is already reported in the literature.[44]–[46] Thus, the recorded FITC-A intensity in sole cell population was considered as autofluorescence of FITC abled laser taken as baseline. In the evaluation, not only forward scattered light could be enough for picking the proper formulation. The results demonstrated that x-axis or forward scattered light determined uptake is not indicating any advance or differences means cells are not granulized and/or swell out during the uptake. If so, they are not demonstrating any difference between each other. On the other hand, site-scattered light versus FITC-A mean indicating that, glioblastoma cells are able to uptake the niosomal nanoparticles in the y-axis manner. It means the uptake should be analyzed in both ways as it is demonstrated in *Figure 9*.

FTIR Profiling for Lyophilized Niosomes and Liposomes

Lyophilized version of each niosome formulation We also performed further structural characterization of these 10 different niosomal formulations and liposome using FTIR after lyophilizing them (see *Supplementary Figure 8*) and obtained their characteristic bands, which are similar to each other.

Confocal Scanning Light Microscopy (CSLM)

FITC-labelled niosomes were incubated for flow cytometry, as optimized 6-hour. Each well-plate prepared for FITC-labelled niosomes were duplicated with the same analytical conditions for CSLM analysis. *Supplementary Figure 10* demonstrated obtained images for promising four niosomal formulations, performed right after the flow cytometer cellular uptake responses.

Cellular Uptake

To utilize the formulated niosomes as improved carrier agents, investigation of their cellular uptake was critical. Establishing the cellular uptake profile of niosomal formulations was a pioneering study, as there were no similar records for niosomes and a comparison with liposomes. The cellular source U-87 was used for the study. 10 different optimized niosome formulations and liposome labelled with fluorescent tracer, FITC, were analyzed by flow cytometry.[47] Incubation time optimization was performed based one of the most reliable physicochemical standings, Opt-10 and the change in FITC-A mean was analyzed by using statistical interface embedded in FlowJo. *Supplementary Figure 11* is demonstrating the only cell population and corresponded FITC-A mean which could be also considered as baseline point for the overall FITC-A mean intensity. Flow cytometer allows three different runs from the same sample, however intraday uptake profiles cannot be considered as one of the replicates for the same experiment. However, their trend could be comparable. Results demonstrated that after 6-hour incubation, slight decrease in FITC-A mean is observed. Thus, all further cellular uptake profiling of niosome and liposome nanocarriers were tested in 6-hour incubation.

The incubation time optimization demonstrated that long incubation period could diminish the uptake could be caused by decreased intensity of FITC since 48-hour cellular viability indicated no toxicity observed for all niosome formulations for U-87 and NFS-60 cell lines. After determination of the most proper incubation time, simultaneous cellular uptake profiling for niosomal and liposomal formulations were tested for 6 – hour. *Figure 9* is demonstrating individual FITC-A mean captured for all testing groups.

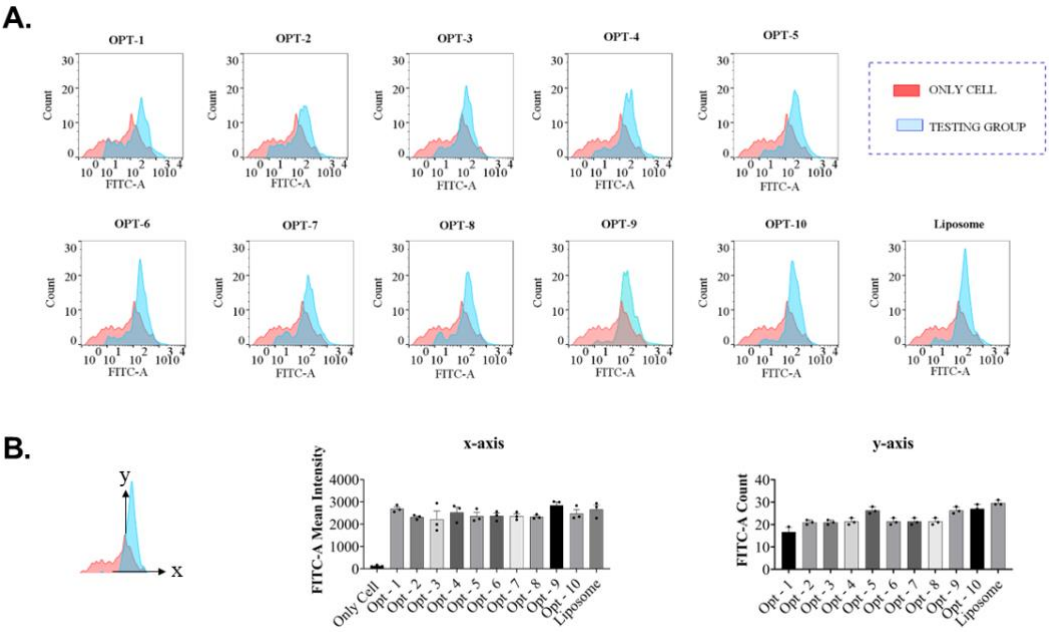


Figure 8. Cellular uptake analysis performed for all niosome and liposome formulations, simultaneously. A. Overlay of histograms to only cell FITC-A gate and their uptake shift were evaluated in the bottom graph. B. FITC-A evaluation based on y-axis and x-axis shift.

Based on all these different analysis for stability, reproducibility, cytotoxicity, and cellular uptake analyses, we found that Opt-6, Opt-8, Opt-9, and Opt-10 were selected as the best formulations among all 10 different formulations (see *Supplementary Figure 9)*. The cellular uptake results showed that even though liposomal and niosomal formulations had similar cellular viability profiles. The study also found that these four niosomal formulations were in the appropriate range in physicochemical characterization, stability, and reproducibility studies for further cellular applications.

4. Conclusion

The aim of this study is to develop different niosomal nanoparticle formulations and characterize their performance in detail to optimize their cellular interactions and safety. The study examines the effects of various process parameters on the physicochemical properties of niosomes, and aims to establish critical ranges for size, polydispersity, and zeta potential. To achieve these optimized conditions, the study investigates 4 different concentrations, 2 different sonication times, 5 different ratios of ingredients, and 2 different types of ingredients. The MODDE software is used to generate 10 different niosomal nanoparticle formulations with a predicted size, sonication time, final concentration, and ingredient amounts. The study also investigates the stability and reproducibility of the optimized formulations. Cellular toxicity and uptake profiles were obtained and compared with liposomes. The results indicate that these optimized niosomal nanoparticle formulations may lead to the

development of more efficient and targeted drug delivery systems. This study could have important implications for the development of new drug delivery strategies that could improve the efficacy and safety of various treatments.

Preliminary screening of process parameters showed that Tween 80 had a more significant impact (***: p-value ≤ 0.001) than Span 60 (*: p-value ≤ 0.05). The use of multiple surfactant types (MSTF) had a greater influence (****: p-value ≤ 0.0001) on the production of niosomes in terms of size (nm), PDI and zeta potential (mV) than SSTF. Final concentration (mM) or sonication time (min) alone did not significantly affect the process. However, two-factor analysis showed that final concentration and sonication time (min) together were the most significant, especially for MSTF (****: p-value ≤ 0.0001) compared to SSTF. The results were then analyzed using MODDE®, a DoE optimization interface, to obtain the best possible niosomal formulations. Logarithmic ordering and preset target range (100-200 nm) resulted in 10 different optimized pseudo-lipid formulations. Then, the similarity between experimental and predicted model was compared by F-score and evaluated as 0.83.

The critical size range (nm) for niosomal nanoparticles was set at 100–200 nm, as this range is considered ideal for drug delivery systems as it allows efficient cellular uptake while minimizing clearance through the reticuloendothelial system.[(48)–(50)] The critical ranges for the PDI and zeta potential were set at 0.2–0.5 and -10 to 10 mV, respectively. Physicochemical characterization revealed that all niosome formulations were within the specified critical range in terms of size (nm), PDI, and zeta potential (mV). Stability testing was performed for 92 days to determine the shelf-life profiles of the formulations for potential drug loading. Stability of size (nm) showed that 5 formulations exhibited slight fluctuations over 92 days, but all niosome formulations were stable in 21 days without aggregation profiles. Seven formulations showed stable PDI without sudden jumps and were within the critical range. All niosome formulations were within the critical range for zeta potential (mV). However, the optimized formulation model required a reproducibility test to determine the most reproducible formulations. Three inter-day experiments were tested for changes in size (nm), PDI, and zeta potential (mV). With the exception of Opt-5 and Opt-7, all niosome formulations were within the critical range in terms of size (nm), PDI, and zeta potential (mV), and for some inter-day experiments, additional statistical confirmation was required beyond evaluating changes in size (nm), PDI, and zeta potential (mV). *Supplementary Table 6* was prepared based on the mean change in each combination between days, and unpaired t-test was performed after a two-way ANOVA test to confirm significant results.

To determine the optimal formulations for improved drug delivery, it was necessary to evaluate their cellular toxicity and cellular uptake profiles. For this purpose, cellular toxicity was assessed using two different cell lines: U-87 (*Figure 7*) and NFS-60 (*Supplementary Figure 5*). The results showed that all niosomal nanoparticle formulations were not toxic to the cells. Moreover, the cellular toxicity of niosomes was compared with that of liposomes, and it was found that both types of nanocarriers had similar cellular viability profiles, ranging between 80-100%. The cellular uptake profiles were determined by optimizing the incubation time, which was set at 6 hours for all formulations. Interestingly, the niosome formulations showed a different FSC-A shift for uptake of FITC-labeled niosomes and liposomes. Four different niosome formulations, namely Opt-6, Opt-8, Opt-9, and Opt-10, showed similar or better cellular uptake compared to liposomes.

The overall analysis and results indicate that the four optimized niosome formulations, Opt-6, Opt-8, Opt-9, and Opt-10, are suitable for drug functionalization due to their favorable physicochemical characterization, stability,

reproducibility, low cellular toxicity, and efficient cellular uptake, which are similar to liposomes. Among these four formulations, Opt-10 appears to be the most promising for advanced drug delivery strategies based on the same analytical perspectives. *Supplementary Figure 9* summarizes the study findings related to physicochemical characterization, stability, and reproducibility.

Supplementary Information

Supplementary information is available online, which includes the following data and profiles for niosomal formulations: Polydispersity and zeta potential profiles of niosomal formulations; Size distributions of different niosome concentrations; Polydispersity and zeta potential profiles of interday experiments; Cellular viability of U87 cells and NFS-60 cells; Standard curves for FITC labeling of niosomes; Flow cytometry data for FITC-labeled niosomes; FTIR analysis of liposome and niosome formulations; Schematic summary for niosome formulation characterization; Confocal images; Flow cytometry data at different incubation times of niosomes.

Tables listing detailed data for the analysis of the effect of sonication time by increased concentration for five different formulations; Correlation analysis of formulation parameters for niosome production; Statistical analysis results for concentration and sonication time parameters based on pre-set criteria of size, PDI, and zeta potential; Two-way ANOVA test results for the effect of the concentration parameter on PDI and zeta potential for each formulation; Stability profiles of optimized formulations in terms of PDI for 21 days, 27 days, 35 days, and 92 days; Stability profiles of optimized formulations in terms of zeta potential for 21 days, 27 days, 35 days, and 92 days; Reproducibility profiles of optimized formulations in size (nm), PDI, and zeta potential (mV); Dynamic Light Scattering data for liposomes.

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References

1. Ahmad H, Arya A, Agrawal S, Dwivedi AK. Novel lipid nanostructures for delivery of natural agents with antioxidant, antiinflammatory and antistroke potential: Perspectives and outcomes. Nanostructures Oral Med. Elsevier Inc.; 2017.

2. Bansal S, Aggarwal G, Chandel P, Harikumar SL. Design and development of cefdinir niosomes for oral delivery. J Pharm Bioallied Sci. 2013;5:318–25.

3. Aparajay P, Dev A. Functionalized niosomes as a smart delivery device in cancer and fungal infection. Eur J Pharm Sci. Netherlands; 2022.

4. Aboubakr EM, Mohammed HA, Hassan AS, Mohamed HB, El Dosoky MI, Ahmad AM. Glutathione-loaded non-ionic surfactant niosomes: A new approach to improve oral bioavailability and hepatoprotective efficacy of glutathione. Nanotechnol Rev. 2021;11:117–37.

5. Ibrahim Bekraki A. Liposomes and niosomes-based drug delivery systems for tuberculosis treatment. In: Kesharwani PBT-NBA for TT editor. Nanotechnol Based Approaches Tuberc Treat. Academic Press; 2020.

6. Mitchell MJ, Billingsley MM, Haley RM, Wechsler ME, Peppas NA, Langer R. Engineering precision nanoparticles for drug delivery. Nat Rev Drug Discov. Springer US; 2021.

7. Fan Y, Marioli M, Zhang K. Analytical characterization of liposomes and other lipid nanoparticles for drug delivery. J Pharm Biomed Anal. 2021.

8. G DB, P VL. Recent advances of non-ionic surfactant-based nano-vesicles (niosomes and proniosomes): a brief review of these in enhancing transdermal delivery of drug. Futur J Pharm Sci [Internet]. SpringerOpen; 2020 [cited 2023 Apr 1];6:1–18. Available from: https://fjps.springeropen.com/articles/10.1186/s43094-020-00117-y

9. van Hoogevest P, Liu X, Fahr A. Drug delivery strategies for poorly water-soluble drugs: the industrial perspective. Expert Opin Drug Deliv [Internet]. Taylor & Francis; 2011;8:1481–500. Available from: https://doi.org/10.1517/17425247.2011.614228

10. Dhoot NO, Wheatley MA. Microencapsulated Liposomes in Controlled Drug Delivery: Strategies to Modulate Drug Release and Eliminate the Burst Effect. J Pharm Sci [Internet]. 2003;92:679–89. Available from: https://www.sciencedirect.com/science/article/pii/S0022354916312011

11. Catarina A, Nuno J, Simoes S. Combination Chemotherapy in Cancer: Principles, Evaluation and Drug Delivery Strategies. Curr Cancer Treat - Nov Beyond Conv Approaches. 2011;

12. Taymouri S, Varshosaz J. Effect of different types of surfactants on the physical properties and stability of carvedilol nano-niosomes. Adv Biomed Res. 2016;5:48.

13. De A, Venkatesh N, Senthil M, Sanapalli BKR, Shanmugham R, Karri VVSR. Smart niosomes of temozolomide for enhancement of brain targeting. Nanobiomedicine [Internet]. SAGE Publications Ltd STM; 2018;5:1849543518805355. Available from: https://doi.org/10.1177/1849543518805355

14. Rezaeizadeh M, Eskanlou A, Soltani H, Pardakhty A, Moshafi MH, Hosseini-Nejad F. Preparation of Stable Clindamycin Phosphate Niosomes by Combination of Sorbitan Esters and their Ethoxylaed Derivatives. J Pharm Innov. 2021;

15. Miatmoko A, Safitri S, Aquila F, Cahyani D, Hariawan B, Hendrianto E, Hendradi E, Sari R. Characterization and distribution of niosomes containing ursolic acid coated with chitosan layer. Res Pharm Sci. 2021;16:660–73.

16. Yeo LK, Chaw CS, Elkordy AA. The effects of hydration parameters and co-surfactants on methylene blue-loaded niosomes prepared by the thin film hydration method. Pharmaceuticals. 2019;12.

17. Aparajay P, Dev A. Functionalized niosomes as a smart delivery device in cancer and fungal infection. Eur J Pharm Sci Off J Eur Fed Pharm Sci. Netherlands; 2022;168:106052.

18. Trucillo P. Drug carriers: Classification, administration, release profiles, and industrial approach. Processes. 2021.

19. Simone EA, Dziubla TD, Muzykantov VR. Polymeric carriers: Role of geometry in drug delivery. Expert Opin Drug Deliv. 2008.

20. Khan I, Saeed K, Khan I. Nanoparticles: Properties, applications and toxicities. Arab J Chem. 2019.

21. De Jong WH, Borm PJA. Drug delivery and nanoparticles: Applications and hazards. Int J Nanomedicine. Dove Medical Press; 2008.

22. Savage DT, Hilt JZ, Dziubla TD. In vitro methods for assessing nanoparticle toxicity. Methods Mol Biol. 2019.

23. Behzadi S, Serpooshan V, Tao W, Hamaly MA, Alkawareek MY, Dreaden EC, Brown D, Alkilany AM, Farokhzad OC, Mahmoudi M. Cellular uptake of nanoparticles: Journey inside the cell. Chem Soc Rev. 2017.

24. Zhu ZJ, Ghosh PS, Miranda OR, Vachet RW, Rotello VM. Multiplexed screening of cellular uptake of gold nanoparticles using laser desorption/ionization mass spectrometry. J Am Chem Soc. 2008;130:14139–43.

25. Singh G, Faruk A, Bedi PMS. Spectral Analysis of Drug Loaded Nanoparticles for Drug-Polymer Interactions. J Drug Deliv Ther. 2018;8:111–8.

26. Kamaraj N, Rajaguru PY, Issac PK, Sundaresan S. Fabrication, characterization, in vitro drug release and glucose uptake activity of 14-deoxy, 11, 12-didehydroandrographolide loaded polycaprolactone nanoparticles. Asian J Pharm Sci. 2017/02/27. Shenyang Pharmaceutical University; 2017;12:353–62.

27. Yaghoobian M, Haeri A, Bolourchian N, Shahhosseni S, Dadashzadeh S. The impact of surfactant composition and surface charge of niosomes on the oral absorption of repaglinide as a BCS II model drug. Int J Nanomedicine. Dove; 2020;15:8767–81.

28. Shah P, Goodyear B, Haq A, Puri V, Michniak-Kohn B. Evaluations of quality by design (QbD) elements impact for developing niosomes as a promising topical drug delivery platform. Pharmaceutics. 2020;12.

29. Tavano L, Muzzalupo R, Picci N, De Cindio B. Co-encapsulation of antioxidants into niosomal carriers: Gastrointestinal release studies for nutraceutical applications. Colloids Surfaces B Biointerfaces. Netherlands; 2014;114:82–8.

30. Noble GT, Stefanick JF, Ashley JD, Kiziltepe T, Bilgicer B. Ligand-targeted liposome design: Challenges and fundamental considerations [Internet]. Trends Biotechnol. Elsevier Current Trends; 2014 [cited 2018 Jan 6]. Available from: http://www.sciencedirect.com/science/article/pii/S0167779913002060

31. Thabet Y, Elsabahy M, Eissa NG. Methods for preparation of niosomes: A focus on thin-film hydration method. Methods [Internet]. 2022;199:9–15. Available from: https://www.sciencedirect.com/science/article/pii/S1046202321001304

32. Khan R, Irchhaiya R. Niosomes: a potential tool for novel drug delivery. J Pharm Investig [Internet]. 2016;46:195–204. Available from: https://doi.org/10.1007/s40005-016-0249-9

33. Silindir-Gunay M, Karpuz M, Ozturk N, Yekta Ozer A, Erdogan S, Tuncel M. Radiolabeled, folate-conjugated liposomes as tumor imaging agents: Formulation and in vitro evaluation. J Drug Deliv Sci Technol. Elsevier; 2019;50:321–8.

34. Hnin HM, Stefánsson E, Loftsson T, Asasutjarit R, Charnvanich D, Jansook P. Physicochemical and Stability Evaluation of Topical Niosomal Encapsulating Fosinopril/γ-Cyclodextrin Complex for Ocular Delivery. Pharmaceutics. 2022;14:1147.

35. Álamo P, Pallarès V, Céspedes MV, Falgàs A, Sanchez JM, Serna N, Sánchez-García L, Voltà-Duràn E, Morris GA, Sánchez-Chardi A, Casanova I, Mangues R, Vazquez E, Villaverde A, Unzueta U. Fluorescent Dye Labeling Changes the Biodistribution of Tumor-Targeted Nanoparticles. Pharmaceutics. 2020;12.

36. Thomsen T, Ayoub AB, Psaltis D, Klok H-A. Fluorescence-Based and Fluorescent Label-Free Characterization of Polymer Nanoparticle Decorated T Cells. Biomacromolecules [Internet]. American Chemical Society; 2021;22:190–200. Available from: https://doi.org/10.1021/acs.biomac.0c00969

37. Joshi S, White R, Sahu R, Dennis VA, Singh SR. Comprehensive screening of drug encapsulation and co-encapsulation into niosomes produced using a microfluidic device. Processes. 2020;8.

38. Owais HM, Baddour MM, El-Metwally HAE-R, Barakat HS, Ammar NS, Meheissen MA. Assessment of the in vitro activity of azithromycin niosomes alone and in combination with levofloxacin on extensively drug-resistant Klebsiella pneumoniae clinical isolates. Brazilian J Microbiol [publication Brazilian Soc Microbiol. 2021;52:597–606.

39. Ag Seleci D, Seleci M, Walter JG, Stahl F, Scheper T. Niosomes as nanoparticular drug carriers: Fundamentals and recent applications. J Nanomater. 2016;2016.

40. Beddoes CM, Case CP, Briscoe WH. Understanding nanoparticle cellular entry: A physicochemical perspective. Adv Colloid Interface Sci. Netherlands; 2015;218:48–68.

41. Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nat Biotechnol [Internet]. 2015;33:941–51. Available from: https://pubmed.ncbi.nlm.nih.gov/26348965

42. Danaei M, Dehghankhold M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A, Khorasani S, Mozafari MR. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics. 2018;10:1–17.

43. De Silva L, Fu J-Y, Htar TT, Muniyandy S, Kasbollah A, Wan Kamal WHB, Chuah L-H. Characterization, optimization, and in vitro evaluation of Technetium-99m-labeled niosomes. Int J Nanomedicine [Internet]. Dove Medical Press; 2019;14:1101–17. Available from: https://pubmed.ncbi.nlm.nih.gov/30863048

44. Li F, Yang M, Wang L, Williamson I, Tian F, Qin M, Shah PK, Sharifi BG. Autofluorescence contributes to false-positive intracellular Foxp3 staining in macrophages: A lesson learned from flow cytometry. J Immunol Methods. 2012;386:101–7.

45. Mosiman VL, Patterson BK, Canterero L, Goolsby CL. Reducing cellular autofluorescence in flow cytometry: An in situ method. Commun Clin Cytom. 1997;30:151–6.

46. Fan Y, Marioli M, Zhang K. Analytical characterization of liposomes and other lipid nanoparticles for drug delivery. J Pharm Biomed Anal [Internet]. 2021;192:113642. Available from: https://www.sciencedirect.com/science/article/pii/S0731708520315284

47. Takikawa M, Fujisawa M, Yoshino K, Takeoka S. Intracellular Distribution of Lipids and Encapsulated Model Drugs from Cationic Liposomes with Different Uptake Pathways. Int J Nanomedicine [Internet]. Dove; 2020;15:8401–9. Available from: https://pubmed.ncbi.nlm.nih.gov/33149583

48. Sun X, Yan X, Jacobson O, Sun W, Wang Z, Tong X, Xia Y, Ling D, Chen X. Improved Tumor Uptake by Optimizing Liposome Based RES Blockade Strategy. Theranostics. Australia; 2017;7:319–28.

49. Li S-D, Huang L. Nanoparticles evading the reticuloendothelial system: role of the supported bilayer. Biochim Biophys Acta. Netherlands; 2009;1788:2259–66.

50. Tang Y, Wang X, Li J, Nie Y, Liao G, Yu Y, Li C. Overcoming the Reticuloendothelial System Barrier to Drug Delivery with a “Don’t-Eat-Us” Strategy. ACS Nano [Internet]. American Chemical Society; 2019;13:13015–26. Available from: https://doi.org/10.1021/acsnano.9b05679