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Modeling and optimization of the niosome nanovesicles using response surface methodology for delivery of insulin

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Niosome is a drug carrier with high stability and biocompatibility that provides higher drug availability, which results in improved therapeutic performance. Despite some promises that show the potential of using niosome for oral delivery of insulin, the effect of multiple factors such as the concentration of the ingredients, mechanical forces, and experimental conditions on final products make the synthesis of insulin-loaded niosomal particle with the proper characteristics very challenging.

In this study, the effects of different factors including the concentration of cholesterol and surfactant, as well as duration of sonication, on zeta-potential, polydispersity index (PDI), and entrapment efficiency (EE%) of the insulin-loaded niosomal vesicles were evaluated and optimum condition was assessed in terms of cytocompatibility and *in vitro* drug release.

The results show that the cholesterol concentration and sonication time have a significant influence on the zeta-potential, whereas the surfactant concentration plays no role. In addition, PDI increased with an increase in cholesterol and surfactant concentration but decreased with increase in the duration of sonication. Furthermore, increasing cholesterol concentration increased EE and increasing the duration of sonication decreased EE.

Considering the effect of different factors, the optimum condition, which is defined as minimum PDI, maximum EE, and zeta potential >±30 mV, occurred while the concentration of the cholesterol and surfactant was 1 M and sonication time was 10 minutes. The zeta potential, PDI, and EE of the optimum insulin-loaded niosomal vesicles

were 36.23±1.04 mV, 0.53±0.07 and 79±2%, which were very close to the predicted values, with the prediction error below 5%.

Finally, the optimum insulin-loaded niosomal vesicles were characterized in different aspects. They displayed a spherical morphology with a low tendency to be aggregated. In addition, they showed good biocompatibility with Caco-2 cells and slow release in simulated intestinal fluid.

INTRODUCTION

In recent years, the pharmaceutical modification has been extensively developed to improve drug absorption and bioavailability. Non-ionic surfactant-based vesicle (Niosome) is a novel drug delivery system formed mostly by cholesterol incorporation as an excipient. Niosome is non-toxic, biocompatible, stable, and capable of encapsulating large quantities of compounds in relatively small volumes of vesicles. Niosome is now extensively investigated as an alternative to liposome as it improves the drawbacks related to liposome, such as high cost, variable phospholipid purity, and chemical instability. In fact, the niosomal system enhances the therapeutic effects of the drug via postponed clearance from the circulation, protection from the biological environment, and restriction of effects to target cells. It also improves oral bioavailability of poorly soluble drugs[1, 2].

Insulin is a peptide drug, which is highly affected by its surrounding environment. Over the last few years, improving bioavailability and anti-diabetic effect of this drug has become a challenge for researchers; such as, the utilization of the oral route instead of the parenteral routes for descending the invasive manners, which results in a more metabolically active surrounding environment. Moreover, the high molecular weight of insulin causes a decrease in permeation across the intestinal epithelium. Thus, it is essential to design and synthesize a system which preserves insulin and increases its permeability in the gastrointestinal tract[3, 4].

So far, in order to improve the bioavailability of oral delivery of insulin, different approaches, such as administration with absorption enhancers, chemical modification, mucoadhesive polymers, and protease inhibitors, have been adopted [5, 6]. Nanostructures, among several functional systems for oral delivery of insulin, facilitate insulin delivery by increasing its permeation across a biological membrane, resulting in potential pharmacological effects. Niosome has been used as a new outstanding candidate for delivery of insulin [3, 7]. Our group showed that the insulin-loaded Trimethyl Chitosan coated niosome prepared using reverse-phase evaporation, exhibited ability for opening the tight cell junctions[3]. In addition, A. Pardakhty, et al exemplified the potential of using niosome as a carrier for oral delivery of insulin in a diabetic rat model [8]. Although all these reports show the potential of using niosome for oral delivery of insulin, neither of them were focused on the effect of different synthesis parameters on the final characteristics of the insulin-loaded niosomal vesicles.

Generally, the most important parameter of the insulin-loaded niosomal vesicles for therapetuticuse, in the pharmaceutical application is entrapment efficiency (EE %). In addition, zeta potential (mV) and polydispersity index (PDI) of vesicles directly affect the stability and drug release rate [7, 9, 10]. Reaching an optimized point in an experiment is very arduous because of the need to adjust different variables and account for the interactions between them, so many experiments are needed, which are time and cost consuming. Since the interaction effect between independent variables (factors) is usually difficult to assess, the real, well-optimized formulation is unreachable. One way to attain this formulation is by using statistical methods; response surface methodology (RSM), one of these statistical methods, is one of the best approaches to overcome these problems and make the optimal condition feasible with the least number of experiments. Therefore, modeling the effect of different synthesis parameters on the final

characteristics of the insulin loaded niosomal vesicles and obtaining the predefined optimized point is the goal of the current study.

The major advantage of using this method to develop pharmaceutical formulations is that the potential factors could be studied simultaneously, systematically, and quickly. By using RSM, the effect of each formulation factor on each response can be estimated, and important factors can be determined according to the statistical analysis.

Different studies have been conducted on different nanostructures using RSM design for the optimization of various form[6, 11-15], but no published work has been found, at least to the best of our knowledge, regarding the formulation and optimization of insulin-loaded niosomes by RSM. Therefore, the present work deals with the modeling and optimization of the synthesis of insulin-loaded niosomes and characterizing the optimum point in terms of morphology, cytotoxicity, and insulin release to prepare the desired system for delivery of insulin.

MATERIALS AND METHOD

2.1. Materials

Insulin was obtained from Exir Pharmaceutical (Lorestan, Iran); Sorbitan monostearate (Span 60) and cholesterol were purchased from Chemical Co. (Germany); Diethyl ether and acetic acid were obtained from Merck Chemical Co. (Germany); Dicetyl phosphate (DCP) was purchased from Sigma (USA); ELISA plates (96 wells) and other plastic ware were obtained from Nunc (MAX-ISORP, Roskilde, Denmark); The cell culture materials including Dulbecco's modified of Eagle's medium (DMEM), fetal calf serum (FCS), and trypsin solution were obtained from Gibco-BRL Life Technologies (Grand Island, NY, USA). Alamar Blue was purchased from Bio-Rad, (Hercules, CA, USA). All other chemicals were of the highest grade commercially available.

2.2. Design of experiment

In this study, a three-level Box–Behnken experimental design, one of the RSM design tools, was specifically selected to evaluate the effect of the independent variables on physicochemical properties of insulin-loaded niosomes, including the zeta potential (mV), PDI, and EE%.

Here the numerical independent variables (factors) were the concentration of cholesterol, surfactant, and the duration of the sonication, which are indicated by (a), (b), and (c) respectively. Three dependent variables (responses) were the zeta potential (mV) of the niosomal nanovesicles, which is a key indicator of the stability of colloidal dispersions, PDI, which is a measure of the heterogeneity of sizes of the particles in the mixture, and EE %, which is a major indicator of drug entrapped into a carrier system. The dependent variables are indicated by (ζ), (δ), and (η) respectively. Table 1 summarizes all the factors and responses with their ranges. The range of the factors was chosen in accordance with the results of preparatory experiments. A total number of 17 experimental runs with five replications of the central point were suggested by the software, which is shown in Table 2. The replication of the central point provided a more precise prediction of experimental error, furthermore, it provided a measure of the adequacy of the model[16].

2.3. Method screening and optimization

2.3.1. Preparation of insulin-loaded niosomal vesicles

Niosome nanovesicles were prepared using reverse-phase evaporation (REV) In accordance with the results of the experimental design, different concentrations of surfactant (span 60) and cholesterol in diethyl ether were mixed. DCP was added to each formulation as a negative

Table 1. Factors and Responses with their ranges

	Name	Unit	Range	
			Low	High
Factor				
a	Cholesterol Con	mol/L	1	2
b	Surfactant Con	mol/L	1	5
c	Duration of sonication	min	10	20
Response			Min	Max
ζ	Zeta potential	mV	-33.0±1.5	-46.1±1.5
δ	PDI		0.513±0.011	0.71±0.02
η	EE	%	70±3	81±3

charge-inducing agent. Then, 3 mg insulin solution in 3 ml phosphate buffer pH 7.4 (0.01 M) was added and mixed well for 1 minute. According to the design, after emulsification, the mixture was sonicated for 10, 15, and 20 minutes in a water bath (10°C) with a bath Sonicator (Fisher Scientific, 40kHz, acoustic power, 185 watts).

The organic solvent (diethyl ether) of the emulsion was eliminated using a rotary evaporator (40°C) at a rotation speed of 50 rpm for about 15 minutes. A high pressure extrusion was carried out by extruding the niosomal suspension through a combination of filters with different pore sizes (1, 0.4, 0.2, 0.1 µm). Five passes were done using the above mentioned filters. To remove free insulin, an ultracentrifuge (25000×g, 4˚C, and 10min) was utilized twice [17]

2.3.2. Encapsulation efficiency of insulin-loaded niosomal vesicles

In order to measure entrapment efficiency (EE) of the insulin-loaded niosomal vesicles, the supernatant was analyzed for determination of non-encapsulated insulin using enzyme-linked immunosorbent assay (ELISA) [18, 19]. EE was calculated based on the following formula (Equation 1) and reported as a percent.

EE= (WT-WF)/WT×100 %,

Where EE is encapsulation efficiency, WT is the total amount of insulin used in the formulation of niosomal vesicle suspensions, and WF is the total amount of free insulin that was found in the supernatant.

2.4. Method validation

2.4.1. Characterization of optimum insulin-loaded niosomal nanovesicles

After prediction of the optimized point by the model, insulin-loaded niosomal nanovesicles were synthesized using the values suggested by the model and characterized in terms of zeta potential, PDI, EE, vesicles morphology, cell compatibility, and *in vitro* release.

2.4.2. Transmission electron microscopy (TEM) of optimum insulin-loaded niosomal nanovesicles

The morphology analysis of the optimum insulin-loaded niosomal nanovesicle was carried out by transmission electron microscopy (TEM). The related size and shape were investigated by negative stain TEM using a Hitachi Transmission Electron Microscope (H-9500, Japan).

2.4.3. Biocompatibility of the optimum insulin-loaded niosomal vesicles

The biocompatibility of optimum insulin-loaded niosomal vesicles was evaluated using the Alamar Blue (AB) assay according to the manufacturer's instructions. Briefly, about 2.5×10^3 Caco-2 cells were seeded in a complete growth media in 48-well plates for 24 h. After that, the cells were incubated with different concentrations of the optimum insulin-loaded niosomal vesicles and compared with cells exposed to complete media and 1% (v/v) Triton X-100 as the positive and negative controls, respectively. After two days of incubation at 37 °C in a humidified atmosphere, AB was added to each well with a final concentration of 10% and incubated for 4 hrs. The well plate was then read in a microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 600 nm.

Table 2. 17 experimental runs with five replications of the central point. (n = 3).

Run	**Factor**			**Responses**		
	a	b	c	ζ	δ	η
	Cholesterol Concentration (mol/L)	Surfactant Concentration (mol/L)	Duration of Sonication (min)	Zeta potential (mV)	PDI	EE (%)
1	1.00	3.00	20.00	-33.04±1.02	0.524±0.002	70±3
2	1.50	3.00	15.00	-41.7±1.3	0.649±0.014	77±4
3	1.50	5.00	10.00	-43.4 ± 0.9	0.653±0.013	76±2
4	2.00	5.00	15.00	-45.3 ± 1.2	0.71±0.02	81±4
5	1.00	1.00	15.00	-35.3 ± 0.9	0.513±0.011	77±3
6	1.50	3.00	15.00	-42.2±1.7	0.639±0.013	76±3
7	1.50	1.00	20.00	-41.2 ± 1.7	0.56±0.02	72±2
8	1.50	1.00	10.00	-43±2	0.578±0.005	78±2
9	1.00	5.00	15.00	-36±2	0.571±0.007	73±2
10	1.50	3.00	15.00	-42.3±1.7	0.63 ± 0.02	76±3
11	2.00	3.00	10.00	-46.1±1.5	0.68 ± 0.03	81±3
12	1.00	3.00	10.00	-37.7±0.9	0.551±0.014	76±3
13	2.00	3.00	20.00	-44±2	0.661±0.012	75±2
14	1.50	3.00	15.00	-43±3	0.629±0.01	77±3
15	2.00	1.00	15.00	-46±1	0.657±0.03	77±3
16	1.50	5.00	20.00	-41.8 ± 1.3	0.64 ± 0.02	73±3
17	1.50	3.00	15.00	-42±2	0.62±0.02	77±4

2.4.4. In vitro release of the insulin from optimum insulin-loaded niosomal vesicles

Drug release from the optimum insulin-loaded niosomal vesicles was evaluated during 14 hours at different time points. To this purpose, the optimum insulin-loaded niosomal vesicles were resuspended in 2 ml of simulated intestinal fluid (SIF) and divided into 4 samples of 500 microliters in Eppendorfs. All the samples were kept in the incubator while on a shaker; then, the supernatant of each sample was taken, and 500 microliters of fresh SIF was added into the samples. The supernatants were evaluated by the previously described ELISA method[18].

2.5. Statistical analysis

Experimental design and statistical analysis were performed using design expert 8 (Stat-Ease Inc., USA). R-square is a statistical measure of how close the data is to the fitted model, but it is not complete. Therefore, adjusted R-squared and predicted R-squared must be considered. Adjusted R-squared is a statistical measure for comparing explanatory power of fitted models that have different numbers of predictor and the predicted R-squared is another statistical measure that indicates how well a model predicts a response. The small difference between the adjusted and predicted R-squared indicates the validity of the model.

In this study, statistical analysis, including the significance of the regression coefficient (T-test), quality of fit of the model (F-test), the lack of fit test, and analysis of variance (ANOVA), were performed in order to evaluate the accuracy of the models. In fact, a fitted model is significant when it has a significant regression and non-significant lack of fit [20, 21].

In addition, The normal probability plot of the model evaluated the normal distribution and the residuals versus predicted plot assessed the assumption of constant variance. The level of significance was determined by a P-value of less than 0.05 ($P < 0.05$) [22].

3. RESULTS and DISCUSSIONS:

3.1. Characteristics of the insulin-loaded niosomal vesicles

According to the software, 17 runs were designed to obtain the best-fitted models. The zeta potential, PDI, and EE of the niosomal nanovesicles were in the range of -33.0 – -46.1 (mV), 0.513 – 0.71, and 70 – 81 %, respectively. All the responses results are listed in Table 2.

3.2. Effect of cholesterol, surfactant concentration, and duration of sonication on the zeta potential of insulin-loaded niosomal vesicles

The zeta potential is an important factor since its value can be related to the stability of nanostructures. The zeta potential increases particle charge, causing particles to repel one another and become more stable against aggregation. Table 2 shows zeta potential of niosomal nanovesicles varies from -33.0±1.5 to -46.1±1.5 (mV) in various concentrations of cholesterol, surfactant, and different durations of the sonication. These zeta potential values are adequately high for electrostatic stabilization. This indicates that niosomes can be suspended in water well, which is critical for their storage and administration.

All the characteristics of the model fitted to the zeta potential of insulin-loaded niosomal vesicles are provided in Table 1. All these values confirm the validity of the model as described in the statistical analysis section. According to the P-value of the coefficient estimate, the cholesterol concentration and sonication time are highly significant in the model (P-value<0.0001), whereas the surfactant concentration is insignificant and played no role in zeta potential of the nanovesicles.

Figure 1 shows the effect of the cholesterol concentration and duration of the sonication on the zeta potential of the niosomal nanovesicles. Zeta potential can be expressed as a function of cholesterol concentration (a) and Duration of sonication (c) which has been shown in Equation 2:

$$\zeta = -20.29 - 28.82a + 0.69c + 7.83a^2 - 0.3ac$$

The negative large coefficient estimate of the cholesterol concentration indicates the most important impact of this factor on the zeta potential of niosomal vesicles. The importance of cholesterol concentration in controlling the zeta potential of nanoparticles was reported in previous studies [23]. The absolute value of the zeta potential increases with increasing cholesterol concentration. This might be due to the effect of cholesterol concentration on the arrangement of surfactant molecules at the interface of the droplet.

Based on the zeta potential model, duration of sonication is the other factor that has a highly significant effect on zeta potential. The reason for this effect could be that with increasing sonication time, the specific surface area increases, which causes the zeta potential of nanovesicles to increase.

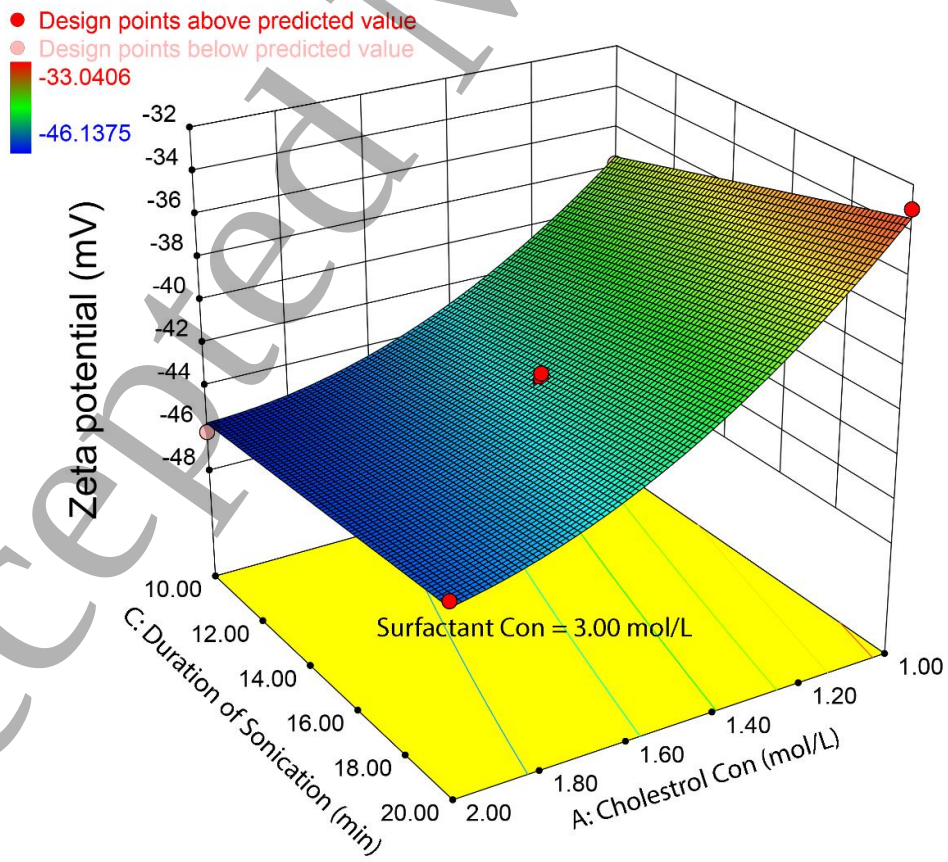

Figure 1. Three-dimensional surface plot of zeta potential based on duration of sonication and cholesterol concentration.

On the other hand, the insignificant P-value of the coefficient estimate of surfactant concentration depicts that this factor has no effect on the zeta potential of the niosomal nanovesicles. The result is consistent with previous reports that showed how the zeta potential of the nanovesicles remains nearly constant over different concentrations of the nonionic surfactant [24, 25]. It has been shown that the zeta potential of the niosomal nanoparticle increases with an increment in hydrophilicity of the nonionic surfactant, which is attributed to the surface energy and the fact that the concentration of the surfactant has no effect on zeta potential[2, 26-28].

Cholesterol concentration and duration of sonication also have an interaction effect on the zeta potential that has appeared as A×C in the model. At all values of duration of sonication, the increment of the cholesterol concentration increases the absolute value of zeta potential (Supplementary 1). Cholesterol, as a sterol compound, could leave interface between lipid and water phase faster as the duration of sonication increased.

At the low level of cholesterol concentration, the changes in zeta potential is slightly higher as the duration of sonication increases (Supplementary 2). It might be attributed to increasing the rigidity of the bilayers membranes of the nanovesicles by increasing the concentration of cholesterol. In fact, the cholesterol content of the bilayer membranes makes them rigid. So, with a low concentration of cholesterol, the sonication force can easily break the larger nanovesicles and causes the change in the specific surface area, leading to higher changes in zeta potential of nanovesicles.

3.3. Effect of cholesterol and surfactant concentration and duration of sonication on the PDI of insulin-loaded niosomal vesicles

The PDI, indicating the size homogeneity of niosomal vesicles, is ranged from 0 to 1. The homogeneity of niosomal vesicles becomes higher as the PDI approaches zero. The minimum amount of PDI is suitable and in this study PDI is ranged from 0.513±0.011 to 0.71±0.02 (Table 2).

Figure 2 (a-c) show the effect of different factors on PDI of the insulin-loaded niosomal vesicles. There is an agreement between adjusted R-squared and predicted R-squared, which proves the statistical significance of the selected model (Table 3). The well-fitted model can be used to navigate the design space. The cholesterol and surfactant concentrations are highly significant in the fitted model (P-value<0.0001), whereas the duration of sonication plays a minor role in PDI of the nanovesicles (P-value = 0.0110).

The equation 3 is model fitted to PDI:

$$\delta = 0.12 + 0.28a + 0.03b + 0.02c - 0.05a^2 - 0.002b^2 - 0.0007c^2$$

The results indicate that the PDI has been increased with increasing cholesterol concentration which is in agreement with previous studies [29-31]. In order to explain the relation between PDI of niosomes with increasing cholesterol content, it is vital to know how the cholesterol is incorporated into the bilayer membrane. Being amphipathic, cholesterol places itself into the bilayer membrane with its hydrophilic head in contact with the aqueous surface, and

the aliphatic chain line up parallel to the hydrophobic chains in the center of the bilayer. Due to the lipophilic nature of cholesterol, an increment in its concentration, will lead to an increase in the hydrophobicity of the bilayer membrane. The vesicle size increases to establish thermodynamic stability, and because of different concentrations of cholesterol in the nanovesicle bilayer membrane, nanovesicles will be shaped in different sizes. That could be the reason for increasing PDI.

In addition, incorporation of cholesterol increases the rigidity of the bilayers and increases the separation between them. It can stabilize the bilayer structure by eliminating phase transition temperature, causing the range of the gel state of vesicles to increase, which results in the bilayer membrane becoming stronger and bilayer microfluidity diminishing. Thus, membrane thickness, particle size, and nanovesicle dispersity will increase. Supplementary 3, shows the influence of the cholesterol concentration as an independent variable on the PDI of the niosomal nanovesicles.

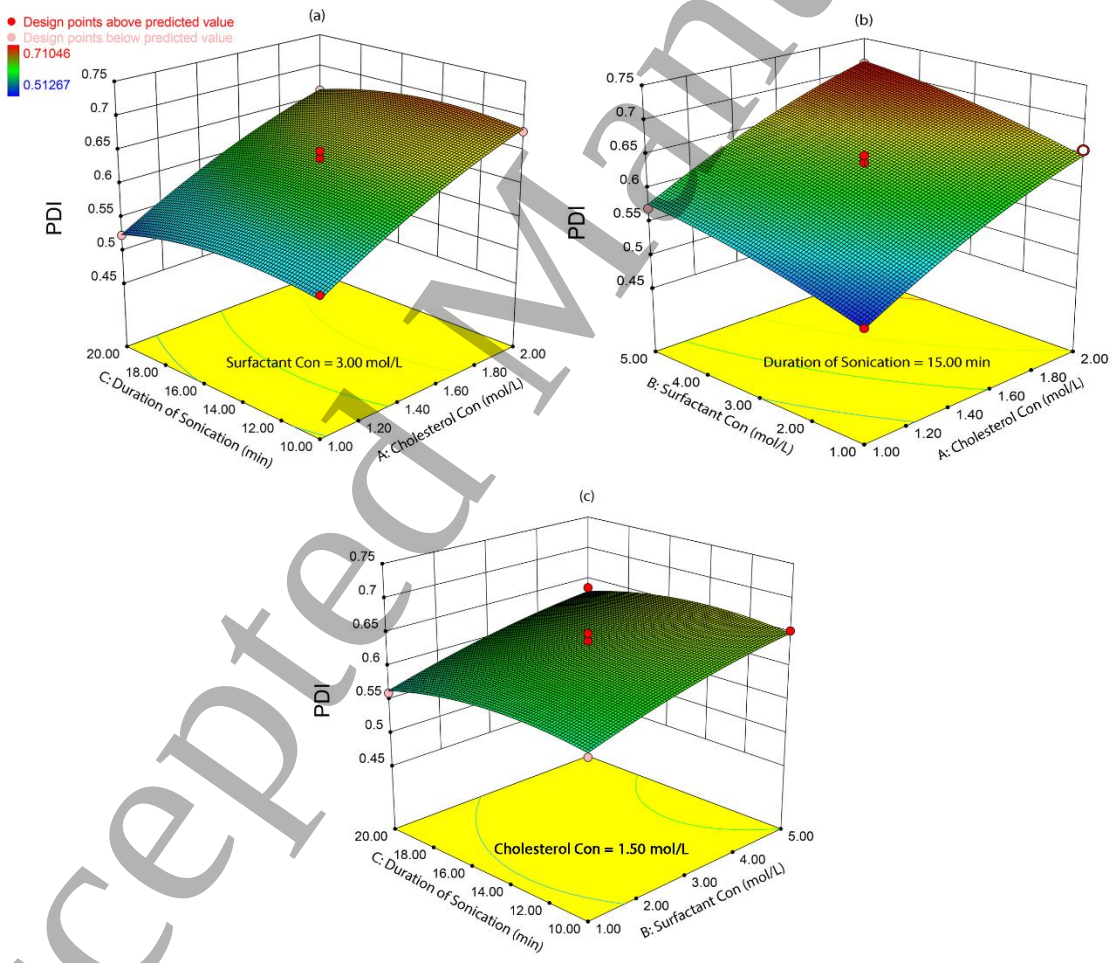

Figure 2. Three-dimensional surface plots of PDI based on (a) duration of sonication and cholesterol concentration (b) surfactant concentration and cholesterol concentration, and (c) duration of sonication and surfactant concentration.

The other factor that affects PDI of the niosomal nanovesicles is the concentration of the surfactant. There is a directly proportional relationship between surfactant concentration and PDI, which might be attributed to the fact that

increasing the surfactant concentration increases the possibility of creating vesicles with different sizes. Thus, the homogeneity of the nanovesicles size will decrease.

Furthermore, there is a reverse relation between sonication time and PDI that is in accordance with the previous studies [32, 33]. Generally, the PDI of the niosomal nanovesicles decreases with increasing exposure to the ultrasound. Ultrasound generates cavitation bubbles with a size near the resonance size of the wavelength of the applied oscillations. . After applying a frequency, the bubbles begin to collapse. These collapses result in a violent implosion that produces high pressure and shock waves. These waves shatter the big vesicles into smaller vesicles and make the vesicles size more homogeneous. Therefore, the PDI of the nanovesicles will decrease. At low concentrations of cholesterol, the vesicular membranes are more flexible to the effect of ultrasound waves, resulting in a bigger change in PDI. However, with increasing cholesterol concentration, the hardness of the membranes is increased, and the membrane will be more resistant to the sonication, thus, the change in PDI is smaller.

Table 3. Characteristics of models fitted to the responses. (Re=Responses, A-R2=Adjust R2, P- R2=Predicted R2, L-fit=Lack of fit, sig=Significant, n-sig=Not significant)

Re	R^2	A- R^2	P- R^2	P-value	L-fit	Significant terms (p-value)
ζ	0.99	0.99	0.97	<0.0001 sig	0.521n-sig	a (<0.0001) c (<0.0001) ac (<0.0036) a^2 (<0.0001)
δ	0.98	0.97	0.96	<0.0001 sig	0.879n-sig	a (<0.0001) b (<0.0001) c (<0.011) a^2 (<0.0195) b^2 (<0.0882) c^2 (<0.0022)
η	0.98	0.96	0.90	<0.0001 sig	0.422n-sig	a (<0.0001) b (0.6634) c (<0.0001) ab (<0.0001) bc (<0.0153) a^2 (<0.0479) c^2 (<0.0004)

3.4. Effect of the cholesterol, surfactant concentration and duration of sonication on the EE of insulin loaded niosomal vesicles

EE is the amount of entrapped drug in nanovesicles, which is reported as a percentage of the total amount of drug used at first. According to Table 2, the EE of the niosomal nanovesicles are suitable and ranged from 70±4 to 81 ± 4. Different conditions of experience provide a different amount of EE. Figure 3 (a-c) show the effect of different factors on EE of the insulin loaded niosomal vesicles.

Equation 4 is model fitted to EE, which can be used to navigate the design space and is highly significant (P-value< 0.0001) (Table 3):

$$\eta = 83.7 - 9.94a - 4.75b + 1.03c + 2.27ab + 0.09bc + 2.57a^2 - 0.06^2$$

According to the P-value of the coefficient estimate, surfactant concentration (b) is an insignificant term (P-value = 0.6634), but it is not eliminated from the model because based on the model, interaction of the surfactant with cholesterol concentration (a) and duration of sonication (c) has an effect on EE of the insulin loaded niosomal vesicles. This effect is denoted as ab and bc in the model equation. Actually, b is a parent term for ab and bc. The parent term should not be omitted even if it does not appear to be statistically significant.

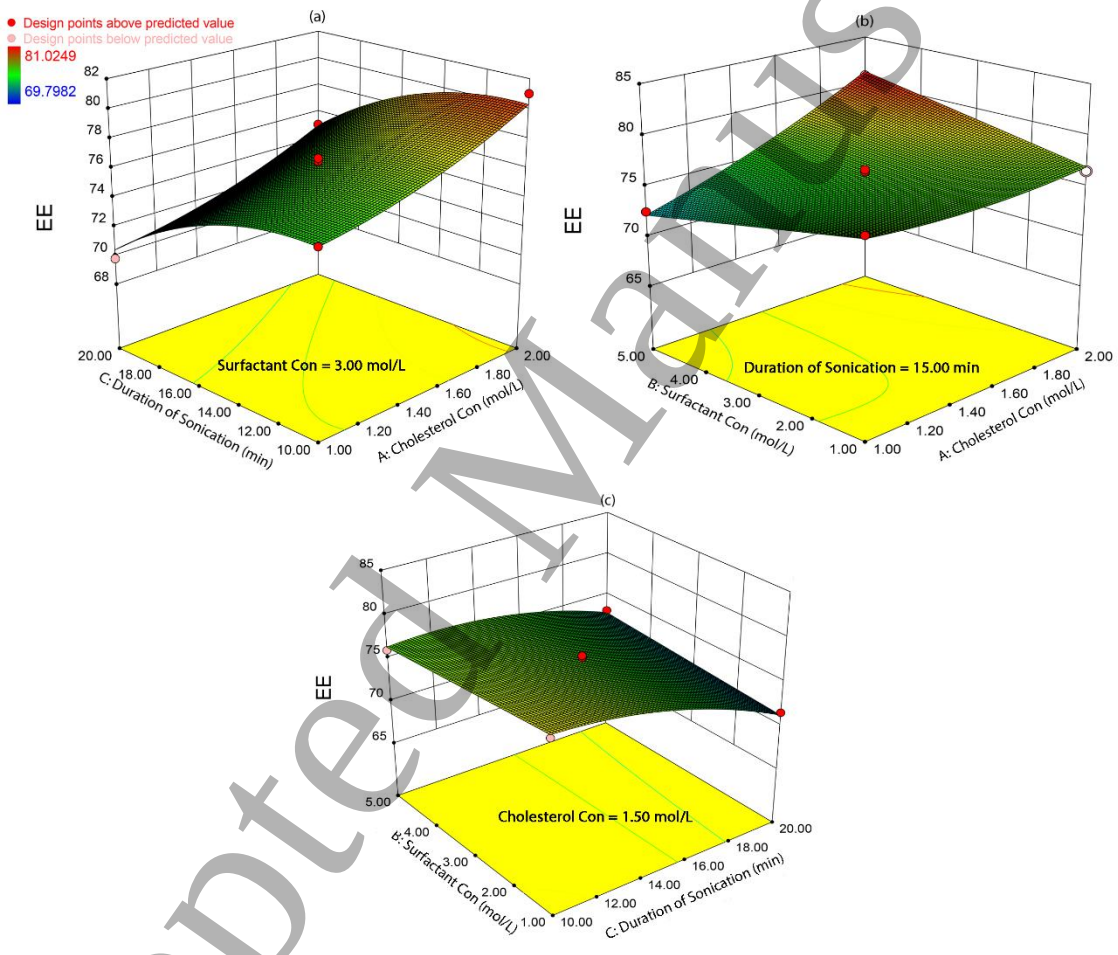

Figure 3. Three-dimensional surface plots of EE based on (a) duration of sonication and cholesterol concentration (b) surfactant concentration and cholesterol concentration, and (c) surfactant concentration and duration of sonication.

The coefficient estimate indicates that cholesterol has a significant influence on the entrapment efficiency of the nanovesicles. This influence is in accordance with the previous report that demonstrates that the inclusion of cholesterol in niosomes increases its EE [34]. Thomas J. McIntosh demonstrated that cholesterol increases the width of phospholipid bilayers by X-ray diffraction[35]. Cholesterol is one of the vital components of the non-ionic surfactant vesicles, namely through its increase in stability and. rigidity, which leads to a reduction in the leakage of water soluble substances through membranes. Thus, an increase in cholesterol concentration of the bilayers results in

a decrease in the release rate of encapsulated drug and an increase in EE. In addition, higher cholesterol concentration increases the interlamellar distance between bilayers in the multilamellar vesicle. This leads to greater overall entrapped volume.

At a high level of surfactant concentration, the EE increased markedly with an increment in the cholesterol concentration. It might be because more material is available to form more vesicles, thus, more volume is available for drug entrapment. For the same reason, the EE increases slightly with an increment in the cholesterol concentration, at a low level of the surfactant concentration (Supplementary 4).

The other factor that has a significant influence on the EE of the nanovesicles is the duration of sonication. The coefficient estimate for the duration of sonication is -2.65 which is approximately at the same range of the cholesterol concentration coefficient estimate. It shows that duration of sonication has an influence on the EE at the same level that cholesterol concentration does. At all concentrations of cholesterol, the relationship between duration of sonication and the EE is indirect. By increasing the former, the latter one will decrease. The relatively large size of the prepared vesicles may be responsible for the relatively high entrapment of the drug. The sonication causes large nanovesicles to breakdown to a smaller size. At high levels of surfactant, the EE decreases slightly; however, in low levels of surfactant concentration, the EE decreases with a higher rate as the duration of sonication increases. It might be attributed to the fact that at a higher concentration of the surfactant, more portion of the drug is entrapped in the vesicles. With a decrease in the surfactant concentration, a higher drug concentration will be out of the vesicles (Supplementary 5).

3.5. Model validation and optimization

In order to characterize an analytical method as robust, it must be able to predict responses with small differences according to the experimental data. Thus, the validity of models was evaluated by experimentally preparing and characterizing the insulin-loaded niosomal vesicles in an optimized point suggested by the model.

To obtain the optimized cholesterol concentration and surfactant concentration, as well as duration of sonication, the optimum point (goals) was set as the point in which minimum PDI and maximum EE would be obtained while zeta potential would be >±30 in which the vesicles are pharmaceutically stable[36]. . Table 4 shows the range of the factors, responses, and goal for each response.

Figure 4 (a-c) show the desirability values for the various conditions. Based on the model prediction, the optimum point will occur in 1 M concentration of cholesterol and surfactant at 10 min sonication. The insulin-loaded niosomal vesicles were prepared using the amount suggested by the software (n=3) and characterized in different aspects.

Table 4. Range of the factors and responses, goal and importance for each response to obtain optimized point. Observed responses, predicted responses and error for validating of the model.OR= Observed response PR= Predicted response

Cholesterol Con (mol/L)	Surfactant Con (mol/L)	Duration of sonication (min)	Zeta potential			PDI			EE		
			Goal >±30 mV			Goal Minimize			Goal Maximize		
			-33.0±1.5 - -46.1±1.5			0.513±0.011 - 0.71±0.02			70±3 - 81±3		
			OR	PR	Error %	OR	PR	Error %	OR	PR	Error %
a = 1	b =1	c = 10	36.23± 1.04	37.355	3.1	0.53± 0.07	0.504	4.3	79±2	78.945	0.0

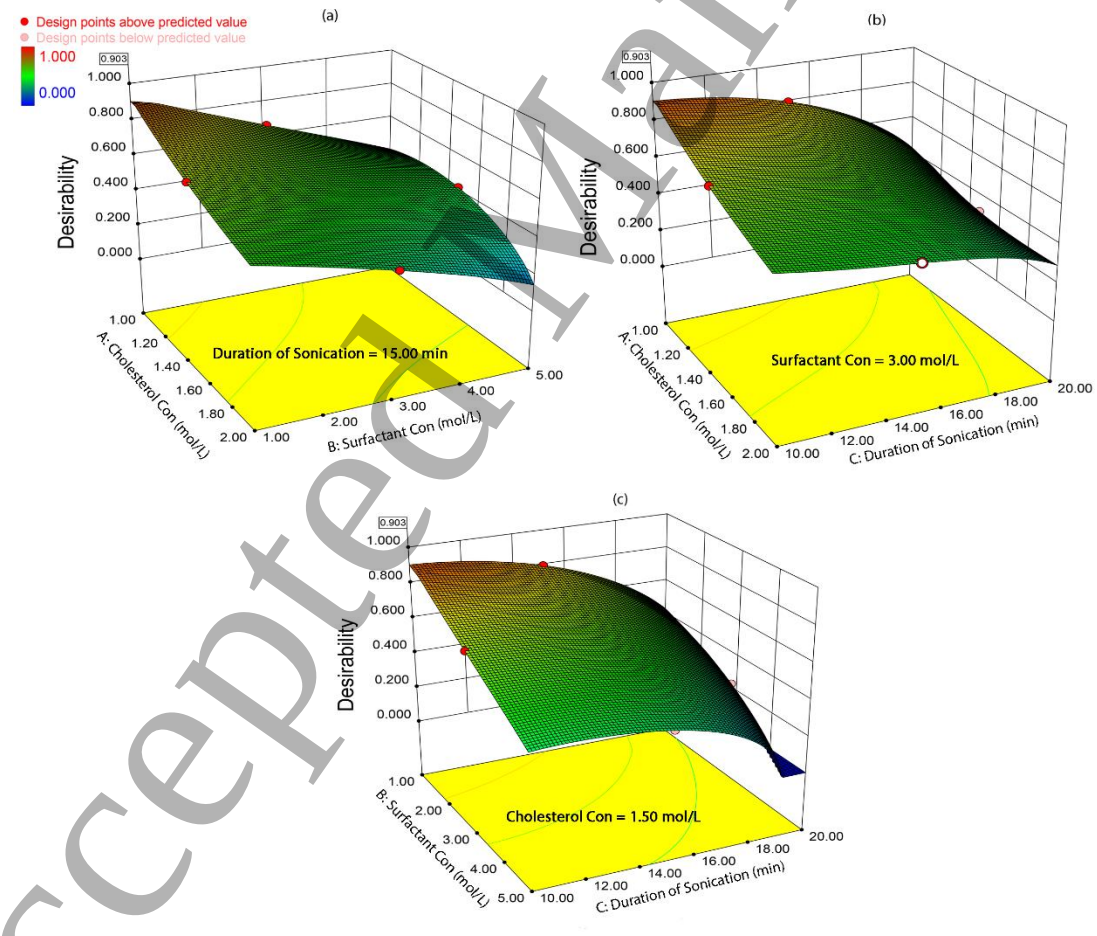

Figure 4 The desirability plots base on (a) cholesterol and surfactant concentration (b) cholesterol concentration and duration of sonication (c) surfactant concentration and duration of sonication.

3.5.1. Encapsulation efficiency, Polydispersity index, and zeta potential of optimum insulin-loaded niosomal vesicles

Table 4 compares the experimental data and predicted responses. The zeta potential, PDI, and EE of the optimum, insulin-loaded niosomal vesicles were 36.23±1.04 mV, 0.53±0.07 and 79±2%, which are very close to predicted values, with the prediction error below 5%. These results indicate that the analytical method was robust, due to a reasonable agreement between predicted and experimental responses.

3.5.2. Transmission electron microscopy (TEM) of optimum insulin-loaded niosomal nanovesicles

Optimum insulin-loaded niosomal vesicles morphology was investigated by TEM imaging. figure 5 shows the spherical shape of the niosome and its low tendency to be aggregated; namely, this can be attributed to ample zeta potential that prevents the aggregation of nanovesicles.

Figure 5. TEM image of optimum insulin loaded niosomal nanovesicles

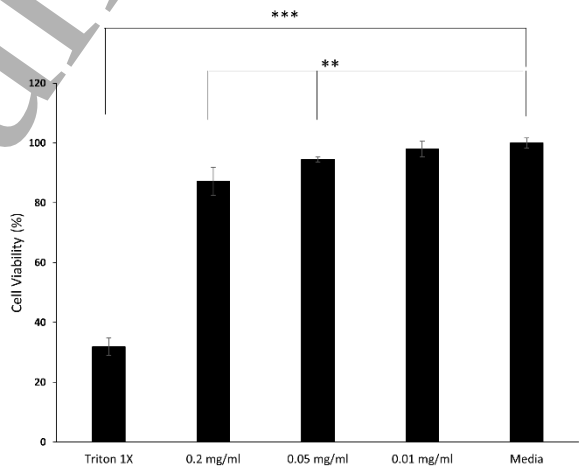

Figure 6. Cell viability of Caco-2 using Alamar Blue after 2 days, expressed as the mean ± SD, n = 3. (P-value<0.01, *** P-value<0.001)**

3.5.3. Biocompatibility of the optimum insulin-loaded niosomal vesicles

Figure 6 shows the cell viability results of the different concentrations of the optimum insulin-loaded niosomal vesicles. The percentage of cell viability for different groups was calculated by dividing the intensity of each treated cell by the cell incubated in complete media. Based on a t test result, there is not any significant differences between cells treated with 0.01 mg/ml and control group. The results show that the insulin-loaded niosomal vesicles with a concentration less than 0.01 mg/ml have no in vitro toxicity to the human epithelial cells.

3.5.4. In vitro release of the insulin from optimum insulin-loaded niosomal vesicles

Drug release profile of insulin from optimum insulin-loaded niosomal vesicles is shown in Figure 7. This graph shows the release of the drug from nanovesicles for 14 hours. Compared to insulin free vesicles, niosomal vesicles release insulin with a very slow rate. There is no significant release from insulin-loaded niosomal vesicles at the initial first 2 Hrs, free insulin, in contrast demonstrated a boost in insulin release in the same time. In fact, the equal molarity of non-ionic surfactant and cholesterol could make the membrane compact and well organized, which might be a reason for the slow insulin release.

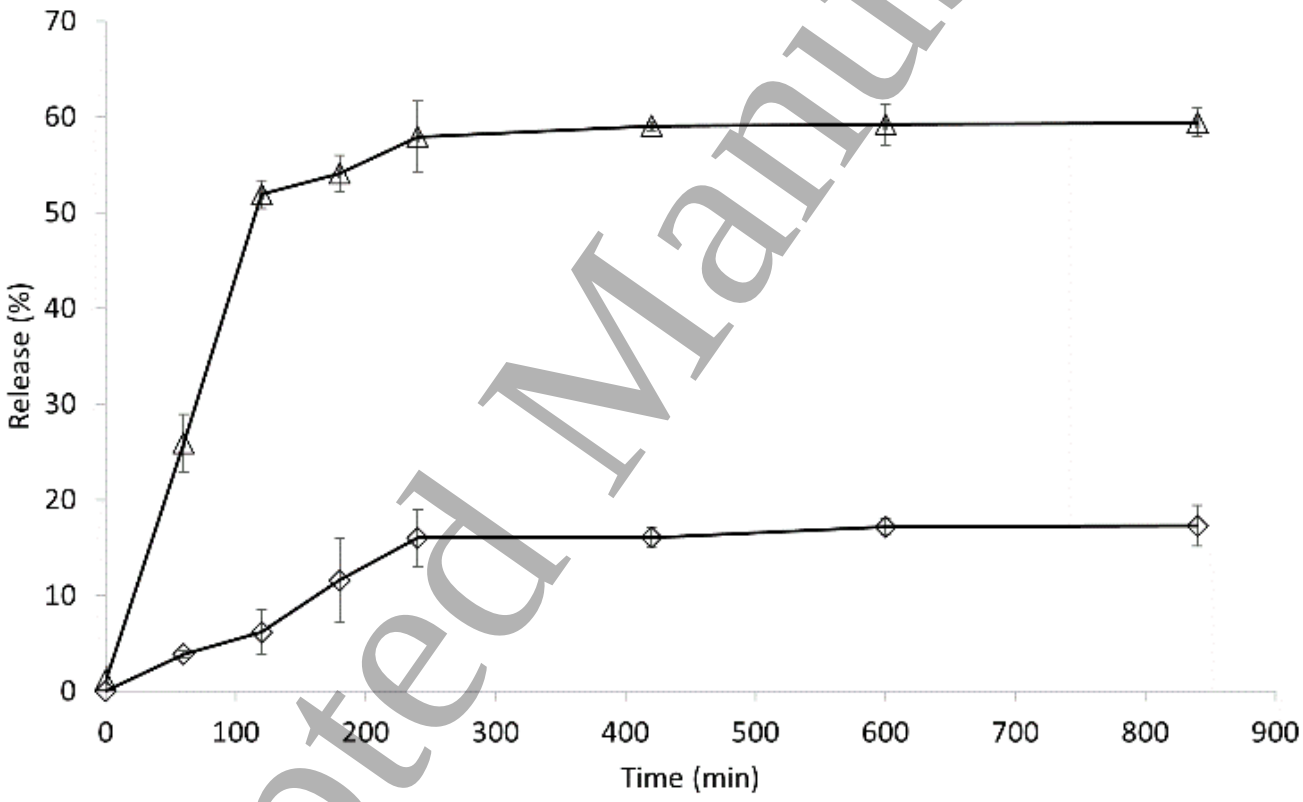

Figure 7. Release of insulin from optimum insulin loaded niosomal nanovesicles in SIF at 37 °C (mean ±SD, n=3) compared to insulin alone.

Δ Optimum insulin loaded niosomal vesicles
◊ Insulin alone.

CONCLUSION

Modeling and optimization of the niosomal nanovesicles for delivery of insulin is a sophisticated and time-consuming procedure. This study presents the development of a formulation to model the synthesis condition of niosomal nanovesicles using RSM. The prepared vesicles were optimized using Box–Behnken response surface experimental design methodology. The three-level, three-factorial Box–Behnken experimental design was applied in the study. The

effects of cholesterol concentration and surfactant concentration, and duration of sonication on zeta potential, PDI, and EE % of insulin-loaded niosomal vesicles were studied.

The results revealed that formulation of the niosomal vesicles with a concentration of 1 mol/L for cholesterol and surfactant under 10 minutes duration of sonication provided an optimum point with the high size homogeneity, maximum EE of drug, and proper zeta potential that is adequately high for electrostatic stabilization. In this study, it was also shown that cholesterol concentration and sonication time influenced zeta potential of the nanovesicles, whereas the surfactant concentration played no role. The increase in cholesterol and surfactant concentration was found to be significantly efficient in increasing the PDI of the niosomal nanovesicles. PDI of the niosomal nanovesicles decreases with increasing duration of sonication. Cholesterol concentration and duration of sonication play a significant role on the EE of nanovesicles; namely, an increase in in cholesterol concentration and duration of sonication causes an increase and a decrease in EE, while the surfactant concentration plays no role in EE of vesicles. It also can be concluded that the influence of the cholesterol concentration on the niosomal nanovesicles characteristics is very vital, and likely to a greater degree than other factors.

In addition, the TEM of the optimum insulin-loaded niosomal vesicles clearly showed the spherical shape of the nanovesicles with the size range of 150 to 300 nm. Furthermore, a cell variability assay depicted the high cell compatibility of the niosomal vesicles in vitro. Finally, slow release of insulin in SIF confirmed the potential of niosomal vesicles to be used as a carrier for slow delivery of insulin.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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