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Acemetacin-Loaded Bilosomal Gel Formulations Prepared Using Different Polymers For Topical Application: Box-Behnken Design For Bilosomes Formulation Optimization And In Vitro Evaluation Of The Formulations

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Abstract

Acemetacin is a poorly water-soluble nonsteroidal anti-inflammatory drug (NSAID), which limits its effectiveness in topical therapeutic applications. This study aimed to enhance the solubility and topical efficacy of acemetacin by developing bilosome-loaded hydrogel formulations. Bilosomes were prepared using the thin-film hydration method and optimized through a Box–Behnken design. The optimized formulation displayed a vesicle size of 137.3 nm, a zeta potential of − 30.1 mV, and an entrapment efficiency of 84.5%. Bilosomes were incorporated into hydrogel bases containing hydroxypropyl methylcellulose (HPMC) or Carbopol. HPMC-based gels exhibited a favorable pH (~ 4) for skin application and were selected for further evaluation. These gels provided sustained drug release for up to eight days. Cytocompatibility testing on L929 fibroblasts using the MTT assay demonstrated cell viability above 90% within the tested concentration range (0.05–2 µg/mL), indicating good biocompatibility. The bilosome-loaded HPMC gel formulation exhibited desirable physicochemical properties, sustained drug release, and excellent cytocompatibility, making it a promising vehicle for topical delivery of acemetacin. Further anti-inflammatory and in vivo studies are recommended to confirm its potential for wound healing applications.

1. INTRODUCTION

Acemetacin (ACIN), chemically known as *1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl* acetic acid carboxymethyl ester, is a glycolic acid ester derivative of indomethacin with antipyretic, anti-inflammatory, and analgesic properties. It has been widely used for the treatment of inflammatory and degenerative disorders [1, 2]. According to the European Pharmacopoeia (EP 10), ACIN is practically insoluble in water [3]. However, it exhibits good solubility in certain organic solvents, such as dimethylformamide (≈ 25 mg/mL), dimethyl sulfoxide (≈ 25 mg/mL), and ethanol (≈ 3 mg/mL). Its solubility in phosphate-buffered saline (PBS, pH 7.2) is approximately 0.5 mg/mL [4]. ACIN acts as a prodrug and undergoes hepatic first-pass metabolism to yield indomethacin, a non-selective cyclooxygenase-2 (COX-2) inhibitor, thereby exerting anti-inflammatory effects. Compared with indomethacin, ACIN is generally assumed to cause less gastric mucosal damage; however, this assumption is supported only by a few small-scale, short-term endoscopic studies, and sufficient clinical evidence is lacking [5, 7]. Chávez-Piña et al. reported that ACIN may exhibit gastric-sparing effects, possibly related to reduced leukocyte adhesion [6].

Wound healing is a complex and dynamic biological process involving numerous tightly coordinated cellular events to repair damaged tissue. Traditionally, it is divided into four main phases: hemostasis, inflammation, proliferation, and dermal remodeling. Cell migration to the wound site is also a crucial component of this process [8, 9].

In adults, the initiation of the inflammatory phase following tissue injury involves the timely activation and recruitment of various cells from both the innate and adaptive immune systems. Inflammatory responses facilitate tissue repair, regeneration, and healing, although they may also lead to fibrotic

outcomes—unlike in fetal tissues, which often heal without fibrosis. One of the critical steps in this process is the timely transition from the inflammatory phase to the proliferative phase. Prolongation or dysregulation of the inflammatory phase can impair subsequent stages of healing and contribute to pathological fibrosis.

Inadequate healing after major injuries—such as trauma, extensive surgery, or severe burns—can result in cutaneous fibrosis. This pathological condition leads to scar formation, which may hinder patient recovery, restrict mobility, and cause cosmetic or functional impairment. As a result, molecular regulators involved in each wound healing phase are being targeted to enhance repair and minimize scarring [10].

During the inflammatory phase, neutrophils and macrophages release large quantities of reactive oxygen species (ROS), which may damage tissues and impair fibroblast and keratinocyte function. Additionally, changes in the wound site's pH are commonly observed during this phase. These factors contribute to delayed healing, particularly in chronic conditions. To mitigate these effects, modified-release systems containing anti-inflammatory agents have emerged as a promising therapeutic strategy [11].

Bilosomes, proposed as an alternative to conventional vesicular systems such as liposomes and niosomes, are bilayer lipid-based vesicular systems stabilized with bile salts. These structures exhibit a high potential for crossing biological membranes due to their unique composition and physicochemical properties [12]. The small vesicle size of bilosomes provides a large surface area, which enhances skin contact time and facilitates drug penetration. In addition, bilosomes offer several advantages, including sustained drug release, improved stability, and reduced side effects [13].

Hydrogels, which closely resemble the natural extracellular matrix, are widely utilized in wound healing applications due to their ability to provide a moist environment that facilitates tissue repair. They can be formulated using either natural (e.g., collagen, gelatin) or synthetic (e.g., methacrylates, polyvinylpyrrolidone) gelling agents. These systems offer several advantages, including skin hydration, support for autolytic wound debridement, and controlled drug release [13]. Hydroxypropyl methylcellulose (HPMC) is a hydrophilic polymer frequently used in modified-release formulations because of its gelling, thickening, and swelling capabilities. It can form clear and stable hydrogels suitable for topical applications [14]. Carbopol®, a cross-linked polyacrylic acid polymer with high molecular weight, is commonly employed as a suspending, thickening, and stabilizing agent in pharmaceutical and cosmetic products. It is valued for its high efficiency, moderate electrolyte tolerance, and ease of use. Various types of Carbopol® (e.g., Ultrez (U), ETD, EZ, 940, 941, 980) are available, each characterized by distinct rheological properties. For instance, Carbopol® 940 and 941 are known for their long wetting times and poor transparency [15]. These polymers can swell up to 1000 times their original volume, forming a mucus-like dispersion [16]. Upon neutralization with agents such as sodium hydroxide (NaOH) or triethanolamine, the ionization of carboxylic acid groups induces negative charges, promoting cross-linking among the swollen polymer chains and enhancing gel strength [17].

Our study aimed to design the ACIN-loaded bilosomal gel formulations to improve analgesic activities of ACIN in the wound healing process. First, we utilized a Box-Behnken design to prepare and optimize ACIN-loaded bilosomes. Thin film hydration method was used to prepare bilosomes formulations. Then, the optimum formulation was incorporated into a gel prepared using Carbopol or HPMC and *in vitro* characterization studies were performed.

2. MATERIALS AND METHODS

2.1. Materials

Acemetacin, cholesterol, and sodium taurocholate were purchased from Sigma-Aldrich (USA). Sunflower phosphatidylcholine (lecithin) was obtained from Shankar (India). Carbopol® Ultrez™ 10 was procured from BF Goodrich (USA), and hydroxypropyl methylcellulose (HPMC) was supplied by Drogsan (Turkey). Dulbecco's Modified Eagle Medium (DMEM) High Glucose, fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased form Sigma-Aldrich (USA). All other chemicals and solvents used were of analytical grade.

2.2. Methods

2.2.1. Box–Behnken experimental design and optimization

A three-level, three-factor Box–Behnken Design (BBD) was used to statistically design and optimize the formulation of ACIN-loaded bilosomes. Design Expert® software (Version 13, Stat-Ease Inc., Minneapolis, MN, USA) was employed to generate 17 experimental runs. The three independent variables included lecithin amount (X1 = A, mg), sodium taurocholate amount (X2 = B, mg), and cholesterol amount (X3 = C, mg), each evaluated at three levels (low, medium, high). The dependent variables (responses) were vesicle size (Y1, nm), zeta potential (Y2, mV), and entrapment efficiency (EE) (Y3, %). Each formulation was prepared and characterized in triplicate (Table 1). The significance of each factor and their interactions on the responses was analyzed using Analysis of Variance (ANOVA), and p-values < 0.05 were considered statistically significant [18].

2.2.2. Preparation of ACIN-loaded bilosomes

First, we prepared ACIN-loaded bilosomes using the thin-film hydration method [19]. Briefly, lecithin, cholesterol, and ACIN (60 mg) were dissolved in ethanol. Ethanol was removed using a rotary evaporator (IKA, RV 3 eco, Germany) at 40 °C under reduced pressure to form a thin lipid film in a round-bottomed flask. The formed lipid film was then hydrated with 20 mL of distilled water containing sodium taurocholate (as a surfactant). The dispersion was sonicated in a bath sonicator (Ultrasonic cleaner WUC-A, Germany) for 10 min to reduce vesicle size. The bilosomal dispersions were stored in a refrigerator (4 °C) until further studies.

Blank bilosomes were prepared the same procedure without ACIN.

2.2.3. Preparation of blank bilosomes or ACIN-loaded bilosomal gel formulations

Formulation No. 15, as presented in Table 1, was chosen as the optimal bilosomal formulation through numerical optimization using response surface methodology (RSM). The selection was driven by maximizing EE%, optimizing vesicle size, and enhancing the absolute zeta potential (ZP), along with achieving a high desirability value within the Box-Behnken design framework. Bilosomal gel formulations were prepared using two different gelling agents (carbopol or hydroxypropyl methyl cellulose). Bilosomes carbopol gels were prepared according to previously described method [20]. Carbopol Ultrez 10 (2.5 or 5%) was dispersed in warm distilled water and mixed at room temperature until a homogeneous mixture on magnetic stirrer at 500 rpm). Then, 20 mL of the optimum formulation (Formulation no. 15; Table 1) or blank bilosomes was added to this mixture and stirred at room temperature until a smooth, homogeneous gel was formed.

In addition, HPMC (2.5 or 5%) was disolved in distilled water and mixed at room temperature until a viscous colloidal solution on magnetic stirrer (at 500 rpm). Then, 20 mL of the optimum formulation (Formulation no. 15; Table 1) or blank bilosomes was added to this mixture and stirred at room temperature until a smooth, homogeneous gel was formed.

2.2.4. In vitro characterization

2.2.4.1. Vesicle size and zeta potential values and morphological properties of bilosomal formulations

The vesicle size measurement for bilosomes formulations was carried out via dynamic light scattering (DLS) technique (Zetasizer Nano ZS; Malvern Instruments, ZEN3600, UK) at room temperature for samples diluted 1:10. We determined the zeta potential values of appropriately diluted samples (1:10) using Zetasizer Nano ZS at room temperature.

The morphological features of the fabricated bilosomal formulations, including blank bilosomes and the optimized ACIN-loaded formulation (Formulation No. 15), were examined using Transmission Electron Microscopy (TEM). The analysis was performed using a Hitachi (Tokyo, Japan) TEM operated at an accelerating voltage of 100 kV. For sample preparation, a drop of the respective bilosomal dispersion was carefully placed onto a carbon-coated copper grid and allowed to air dry at room temperature. Images were subsequently acquired at appropriate magnifications to ensure accurate visualization of vesicle morphology [21].

2.2.4.2. Texture analysis and pH measurement for bilosomal gel formulations

We used a Texture Analyser (TexturePro CT V1.9 Build 35, Brookfield Engineering Labs. Inc.) equipped with a 1.5 kg load cell to determine the texture properties (adhesiveness, cohesiveness, and hardness) of

the bilosomal gel formulations. Briefly, a 10-mm (diameter) cylindrical probe was compressed into the gel (10 g) for a distance of 10 mm (at a speed of 2 mm/sec) and redrawn. Three measurements were made at 25 ± 0.5°C for each sample.

The pH measurements for ACIN-loaded bilosome and bilosomal gel formulations were performed via a pH meter (“Mettler Toledo FiveGo, Switzerland). Three measurements were made at at 25 ± 1°C for each sample [22].

2.2.4.3. EE% values for bilosomal formulations and bilosomal gel formulations

To determine the percentage of EE% for ACIN-loaded bilosomes or ACIN-loaded bilosomal gel formulation, the method described by Akaki et al. was used with modifications [23]. ACIN-loaded bilosomes (0.5 g) or ACIN-loaded bilosomes-based gel formulation (0.5 g) were mixed with 100 mL of mobile phase (ACN:%1 formic acid; 60:40) and mixed for 1 h on magnetic stirrer (500 rpm) at room temperature. Then, the mixture was filtered through a membran filter (pore size: 0.45 µm) and analyzed in HPLC. The experiments were made triplicate. The EE% was calculated by using the following equation= (Amount of ACIN in the formulation/Total amount of ACIN added)x100.

2.2.4.4. In vitro release study

The prepared ACIN-loaded bilosomes or ACIN-loaded bilosomes-based gel formulations (1 g) were filled into the dialysis bags (cut off: 12–14 kDa). Then, they were put into 100 mL of release medium (PBS-pH 7.4 and ethanol; 70:30 v/v; for sink condition) and regularly stirred at 150 rpm at 32 ± 1 °C. Samples (1 mL) are collected at predetermined time intervals (0, 0.25, 0.5, 1, 2, 3, 4, 6, 8 h) and and replaced with same volume fresh release medium. The samples were filtered through a membran filter (pore size: 0.45 µm) and analyzed in HPLC. The addition of organic co-solvents to the dissolution/release medium can increase drug solubility. There are studies in the literature on the use of co-solvents to increase drug solubility. In these studies, ethanol was used at various rates (10%, 40%, etc.) to ensure that sink condition was maintained [24].

2.2.5. Cytotoxicity assay

In this study, L929 human fibroblast cell line (ATCC, USA), a beneficial model for assessing toxicity from dermal exposure, was used. The cells were cultured in high-glucose DMEM supplemented with 10% FBS, 1% non-essential amino acids, and 1% penicillin-streptomycin. When the cells reached 90% confluence in culture flasks, they were passaged using Trypsin-EDTA.

Cytotoxicity of formulations was determined with the MTT assay. Briefly, L929 cells were seeded into 96-well microplates (10,000 cells/well). After overnight incubation at 37°C, the growth medium was replaced with fresh medium containing from 0.05, 0.1, 0.25, 0.5, 1, and 2 µg/mL blank bilosomes, ACIN-loaded bilosomes, blank bilosomal gel formulation prepared using HPMC (2.5%) (B-bilosomal HPMC 2.5 gel), and ACIN-loaded bilosomal gel formulation prepared using HPMC (2.5%) (ACIN-bilosomal HPMC

2.5 gel in 96 well plates. The cells were incubated for additional 24 h. The final concentration of DMSO in solvent control and dilutions was 0.1%. MTT was then added to a final concentration of 0.5 mg/mL and the cells incubated at 37°C for 3 h. The medium was removed, and formazan crystals were dissolved in DMSO (Sigma Aldrich, USA). The optical density (OD) of the solution in each well was measured at 570 nm (the formazan absorption peak) by a microplate spectrophotometer (Varioskan LUX Multimode Microplate Reader, Thermo Fisher). The viability of cells was determined by comparing formazan concentrations of the treated cells with those of untreated control cells [24]. Each treatment group consisted of six replicate wells. The experiments were repeated three times.

2.2.6. Statistical analysis

Data were analyzed using GraphPad Prism software (version 10.2.3). Following the Shapiro-Wilk normality test, one-way ANOVA was performed, followed by Tukey's post hoc test. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

A three-level, three-factor Box-Behnken design was used to prepare and optimize ACIN-loaded bilosomes. A total of 17 formulations were obtained by varying three formulation parameters (amount of lecithin, amount of sodium taurocholate and amount of cholesterol). The effect of these formulation variables on the formulation characteristics (zeta potential, vesicle size and EE%) is presented in Table 1.

Table 1
Box-Behnken Design and the obtained responses.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Run	X1 = A:Lecithin	X2 = B:Sodium taurocholate	X3 = C:Cholesterol	Y1 = Vesicule size (n = 3)	Y2 = Zeta potential (n = 3)	Y3 = EE (n = 3)
	Mg	Mg	mg	Nm	mV	%
1	500	17.5	30	181.9 ± 0.11	-28.9 ± 0.02	50.4 ± 0.11
2	300	17.5	22.5	169.5 ± 0.12	-45.9 ± 0.02	64.8 ± .0.09
3	300	10	30	174 ± 0.11	-41.4 ± 0.01	67 ± 0.12
4	500	17.5	15	145.5 ± 0.12	-32.7 ± 0.01	62.8 ± 0.10
5	100	17.5	15	560 ± 0.13	-55.3 ± 0.02	49.5 ± 0.09
6	500	25	22.5	174.7 ± 0.12	-39 ± 0.02	78.9 ± 0.09
7	300	17.5	22.5	116.9 ± 0.12	-49.1 ± 0.01	63.2 ± 0.10
8	300	25	15	105.5 ± 0.11	-43.9 ± 0.02	69.8 ± 0.12
9	300	17.5	22.5	141.4 ± 0.11	-50.2 ± 0.02	68.1 ± 0.11
10	100	17.5	30	231 ± 0.12	-54.9 ± 0.01	49.6 ± 0.12
11	300	17.5	22.5	182 ± 0.11	-35.2 ± 0.01	63.5 ± 0.11
12	300	25	30	127.4 ± 0.12	-37.6 ± 0.02	82.7 ± 0.10
13	100	10	22.5	227 ± 0.12	-56.7 ± 0.01	67.8 ± 0.11
14	300	17.5	22.5	214.9 ± 0.11	-55.1 ± 0.02	52.3 ± 0.09
15	500	10	22.5	137.3 ± 0.12	-30.1 ± 0.02	84.5 ± 0.10
16	100	25	22.5	373.7 ± 0.12	-58.6 ± 0.02	80.1 ± 0.11

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
17	300	10	15	159.7 ± 0.11	-64.4 ± 0.01	60.5 ± 0.12

3.1. Experimental design and statistical analysis

The results from the Box-Behnken design were analyzed using ANOVA to determine the significance of the independent variables.

Effect of independent variables on vesicular size: The quadratic model developed for vesicle size was statistically significant (p = 0.0444, Table 2).

Table 2
Statistical analysis for quadratic model to evaluate the effects of formulation variables on the vesicle size of ACIN-loaded bilosome formulations.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.645E + 05	9	18273.90	3.86	0.0444	Significant
X1 = A-Lecithin	70744.41	1	70744.41	14.94	0.0062	
X2 = B-Sodium Taurocholate	867.36	1	867.36	0.1831	0.6816	
X3 = C-Cholesterol	8217.62	1	8217.62	1.74	0.2292	
AB	2986.62	1	2986.62	0.6306	0.4532	
AC	33379.29	1	33379.29	7.05	0.0327	
BC	14.44	1	14.44	0.0030	0.9575	
A^2	42605.69	1	42605.69	9.00	0.0200	
B^2	5876.14	1	5876.14	1.24	0.3021	
C^2	833.24	1	833.24	0.1759	0.6875	
Residual	33153.24	7	4736.18			
Lack of Fit	27483.43	3	9161.14	6.46	0.0516	not significant
Pure Error	5669.81	4	1417.45			
Cor Total	1.976E + 05	16				

Among the tested variables, the concentration of lecithin (X1 = Factor A) demonstrated the most significant negative effect on vesicle size ($p = 0.0062$), indicating that increasing lecithin concentration led to smaller vesicles within the studied range. At lower concentrations (100 mg), the vesicles were significantly larger, ranging from 227 nm to 560 nm (Table 1), suggesting poor structural compactness and weak bilayer organization. However, as the lecithin concentration increased to medium (300 mg) and high (500 mg) levels, vesicle sizes consistently decreased, falling between 105.5 nm and 214.9 nm (Table 1). This reduction in size suggests that higher lecithin concentrations facilitate improved bilayer packing, resulting in smaller and more stable vesicles with better membrane organization. The quadratic term of lecithin (A^2) was significant ($p = 0.0200$), highlighting a non-linear relationship between lecithin concentration and vesicle size. This suggests that beyond a certain concentration, further increases in lecithin lead to diminishing effects on vesicle size reduction (Fig. 1).

Figure 2 demonstrates an interaction between lecithin and cholesterol. At low lecithin levels, increasing cholesterol from 15 mg to 30 mg reduces vesicle size significantly. However, at higher lecithin concentrations, changes in cholesterol have less impact on vesicular size. This confirms the significant interaction term (AC, $p = 0.0327$) (Table 2), suggesting cholesterol helps control vesicle size particularly when lecithin amount is low. The downward-sloping curvature reflects the combined stabilizing roles of both lipids. These results emphasize the critical role of lecithin concentration and its interaction with cholesterol in modulating bilosome size.

At a constant high lecithin concentration (500 mg), the 3D surface plot (Fig. 3) shows that increasing sodium taurocholate (X2 = B) from 10 mg to 25 mg slightly reduces vesicular size, as indicated by a gentle downward slope along the B-axis, while increasing cholesterol (X3 = C) from 15 mg to 30 mg slightly increases the size, shown by a mild upward slope along the C-axis. Overall, the surface remains relatively flat with minor variations, suggesting that while sodium taurocholate and cholesterol do influence vesicle size, their effects are modest compared to the dominant size-reducing impact of high lecithin.

Effect of independent variables on zeta potential: The 2FI (Two-Factor Interaction) model fitted for zeta potential was statistically significant ($p = 0.0173$; Table 3).

Table 3
ANOVA summary for the 2FI model assessing the influence of formulation variables on the zeta potential of ACIN-loaded bilosome formulations.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1371.31	6	228.55	4.57	0.0173	significant
X1 = A-Lecithin	1123.38	1	1123.38	22.48	0.0008	
X2 = B-Sodium Taurocholate	22.78	1	22.78	0.4560	0.5148	
X3 = C-Cholesterol	140.28	1	140.28	2.81	0.1247	
AB	12.25	1	12.25	0.2452	0.6312	
AC	2.89	1	2.89	0.0578	0.8148	
BC	69.72	1	69.72	1.40	0.2648	
Residual	499.63	10	49.96			
Lack of Fit	278.97	6	46.49	0.8428	0.5953	not significant
Pure Error	220.66	4	55.16			
Cor Total	1870.93	16				

The amount of lecithin (X1 = Factor A) was found to have a highly significant impact on zeta potential ($p = 0.0008$). Sunflower lecithin contains primarily phosphatidylcholine. It is a source of phosphatidylinositol, and phosphatidylethanolamine. Phosphatidylcholine contains a choline moiety with a positive charge on the trimethyl-amino group and a negative charge on the phosphate group. Phosphatidylethanolamine has amino and phosphate groups, while phosphatidylinositol contains hydroxyl groups. Bot et al. reported that liposomes prepared using soy lecithin had a negative zeta potential values [26]. In our study, the prepared bilosomes formulation had negative zeta potential values (Fig. 4–6; Table 1).

Figure 4 illustrates the impact of lecithin and cholesterol on zeta potential when sodium taurocholate is held constant at a low concentration (10 mg). As lecithin concentration increased, the absolute zeta potential value decreased (Fig. 4), suggesting a reduction in negative surface charge density (Fig. 4). Similarly, increasing the cholesterol concentration from 15 to 30 mg also resulted in lower absolute zeta potential values (i.e., becoming less negative) (Fig. 4), but this effect was insignificant ($p = 0.1247$; Table 3).

Figure 6 shows how cholesterol and sodium taurocholate influence zeta potential when lecithin is maintained at a high concentration (500 mg). At this high lecithin level, increasing cholesterol from 15

mg to 30 mg consistently results in the zeta potential becoming less negative (shifting from greenish-yellow towards orange/red). In contrast, increasing sodium taurocholate from 10 mg to 25 mg caused the zeta potential to become more negative (moving from the orange/red area towards the yellow/green area).

Effect of independent variables on EE%: The EE data were best described by a statistically significant quadratic model (p = 0.0327, Tablo **4**).

Table 4
ANOVA results for the quadratic model evaluating the effects of formulation variables on the EE% of ACIN-loaded bilosome formulations

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1830.16	9	203.35	4.36	0.0327	significant
X1 = A-Lecithin	132.85	1	132.85	2.85	0.1355	
X2 = B-Sodium Taurocholate	102.96	1	102.96	2.21	0.1811	
X3 = C-Cholesterol	6.30	1	6.30	0.1350	0.7242	
AB	109.20	1	109.20	2.34	0.1700	
AC	39.06	1	39.06	0.8366	0.3908	
BC	10.24	1	10.24	0.2193	0.6538	
A^2	0.5609	1	0.5609	0.0120	0.9158	
B^2	1154.67	1	1154.67	24.73	0.0016	
C^2	336.52	1	336.52	7.21	0.0313	
Residual	326.85	7	46.69			
Lack of Fit	184.74	3	61.58	1.73	0.2979	not significant
Pure Error	142.11	4	35.53			
Cor Total	2157.01	16				

The main effects of lecithin (X1 = A), sodium taurocholate (X2 = B), and cholesterol (X3 = C), as well as their binary interactions (AB, AC, BC), were not statistically significant (p > 0.1; Table 4). However, the quadratic terms of sodium taurocholate (B^2) and cholesterol (C^2) were highly significant (p = 0.0016 and p = 0.0313, respectively; Table 4), indicating a strong non-linear effect of these two components on ACIN encapsulation into bilosomes.

The combined impact of lecithin and cholesterol on EE% when sodium taurocholate is maintained at a medium level (17.5 mg) is shown in Fig. 8. Increasing the lecithin concentration from 100 mg to 500 mg resulted in a improvement in EE% values, as indicated by the upward slope of the surface along the lecithin axis. Cholesterol also exhibited a non-linear effect; EE% values were generally lower at the lowest cholesterol level (15 mg), increased significantly as cholesterol increased to a medium level (around 22.5 mg), and then appeared to level off or slightly decreased at the highest level (30 mg) (Fig. 8), forming a ridge-like shape along the cholesterol axis. Under these conditions (medium level for sodium taurocholate), the highest EE% values are achieved with acombination of high lecithin and moderate (around 22.5 mg) to high cholesterol amounts, while the lowest EE% values are seen at low lecithin and low cholesterol.

Figure 9 shows the effects of sodium taurocholate and cholesterol on EE% when lecithin is at a high concentration (500 mg). At this high lecithin level, increasing cholesterol from 15 mg to 30 mg generally led to higher EE%, as the surface slopes upwards along the cholesterol axis. Sodium taurocholate showed a U-shaped response, with higher EE% values observed at both the low (10 mg) and high (25 mg) levels compared to the intermediate level (around 17.5 mg) (Fig. 9). As a result, the combination of high lecithin (500 mg) with high cholesterol and low or high sodium taurocholate resulted in the highest EE% values.

3.2. Formulation optimization

Based on the analysis, the Box-Behnken design facilitated the identification of an optimal formulation composition aimed at minimizing vesicle size, achieving a favorable zeta potential (negative for stability), and maximizing EE%. Formulation number 15, composed as specified in Table 1, experimentally yielded a vesicle size of 137.3 ± 0.12 nm, a zeta potential of -30.1 ± 0.02 mV, and an EE of 84.5%. The desirability plot (Fig. 10) and the overlay plot (Fig. 11) visually represented the design space meeting these optimal characteristics, guiding the selection of the final formulation. These values met the optimization criteria, confirming the suitability of this composition as the optimized ACIN-loaded bilosomal formulation.

In addition, the vesicle size, zeta potential values for the the optimum blank bilosomal formulation were 160 ± 10.12 nm, and − 24.5 ± 0.12 mV (n = 3), respectively.

3.3. Morphological evaluation of the optimum bilosome formulations

TEM images were obtained to evaluate the morphology of the optimum bilosome formulations (Fig. 12**)**. In Fig. 12**-A**, the blank bilosomes appeared as nanoscale nearly spherical vesicles with smooth surfaces. The optimized ACIN-loaded bilosome formulation (Formulation No. 15, Fig. 12**-B1** and **B2**) was nano-sized, spherical and had distinct boundaries. The observed vesicle sizes of ACIN-loaded bilosome formulation in TEM images typically ranged from 130 to 265 nm.

3.4. Texture analysis and pH measurement for bilosomal gel formulations

In this study, gel formulations containing the optimum bilosomal formulation were also prepared using Carbopol® Ultrez™ 10 or HPMC and *in vitro* characterized. Firstly, the mechanical properties such as adhesiveness, cohesiveness, and hardness of the ACIN-loaded bilosomal gel formulations prepared using Carbopol or HPMC (2.5 or 5%) were investigated using texture profile analysis (Table 5).

In addition, the pH values were determined for ACIN-loaded bilosomes (the optimum formulation) and also ACIN-loaded bilosomal gel formulations prepared using Carbopol or HPMC (2.5 or 5%). The pH values are given in Table 6.

Table 5
The Mechanical Properties of ACIN-loaded bilosomal gel formulations prepared using Carbopol or HPMC (2.5 or 5%) (Mean ± SD; n = 3)

	Carbopol 2.5 gel	Carbopol 5 gel	HPMC 2.5 gel	HPMC 5 gel
Hardness (N)	0.180 ± 0.010	1.250 ± 0.078*	0.030 ± 0.000	0.047 ± 0.006
Adhesiveness (mJ)	0.993 ± 0.068	3.490 ± 0.010*	0.057 ± 0.006	0.220 ± 0.026*
Cohesiveness	0.873 ± 0.015	0.770 ± 0.017	0.667 ± 0.095	0.517 ± 0.119

Carbopol 2.5 gel and Carbopol 5 gel: ACIN-loaded bilosomal gel formulations prepared using Carbopol (2.5 or 5%); HPMC 2.5 gel and HPMC 5 gel: ACIN-loaded bilosomal gel formulations prepared using HPMC (2.5 or 5% (*: $p < 0.05$)

Table 6
The pH values of ACIN-loaded bilosome formulation and ACIN-loaded bilosomal gel formulations prepared using Carbopol or HPMC (2.5 or 5%) (Mean ± SD; n = 3)

Formulation	pH
ACIN-loaded bilosomes	3.46 ± 0.015
Carbopol 2.5 gel	3.76 ± 0.053
Carbopol 5 gel	3.81 ± 0.045
HPMC 2.5 gel	4.07 ± 0.026
HPMC 5 gel	4.08 ± 0.031

ACIN-loaded bilosomes: ACIN-loaded bilosome formulation (optimum formulation); Carbopol 2.5 and Carbopol 5: ACIN-loaded bilosomal gel formulations prepared using Carbopol (2.5 or 5%); HPMC 2.5 and HPMC 5: ACIN-loaded bilosomal gel formulations prepared using HPMC (2.5 or 5%)

3.5. EE% values for ACIN-loaded bilosomal gel formulations and *in vitro* release studies

Moreover, EE% values were determined for ACIN-loaded bilosomal formulations prepared using Carbopol or HPMC (2.5 or 5%). While the EE% values for ACIN-loaded bilosomal gel formulation prepared using Carbopol (2.5 or 5%) were 86 ± 0.12% and 86 ± 0.15 (n = 3), ACIN-loaded bilosomal gel formulation prepared using HPMC (2.5 or 5%) were determined as % 89 ± 0.13% and 88 ± 0.14 (n = 3), respectively.

In our study, the in vitro release studies were also performed for the optimum bilosome formulation and the bilosomal gel formulations prepared using HPMC (2.5 or 5%) in PBS-pH 7.4 and ethanol (70:30 v/v) mixture. The results of the release studies are presented in Fig. 13**-A** and **13-B.**

3.6. The results of cytotoxicity assay

Cell viability values obtained after 24 h exposure of cells (n = 6) to the test substance at specified concentrations (0.05, 0.1, 0.25, 0.5, 1, and 2 µg/mL) are presented in the Fig. 14. The percentage of viability of cells treated with the formulations was calculated by comparing it with that of control cells whose viability was taken as 100%.

The viability of cells treated with the above-mentioned formulations ranged from 87.76–106.9% (Fig. 14). The viability of cells was about 90% and above in all tested samples, indicating that both blank or ACIN-loaded bilosomes and bilosomal HPMC 2.5 gel formulations had no cytotoxic effect in the indicated concentration range. Although slight decreases in cell viability were observed at 2 µg/mL in ACIN-loaded bilosomal HPMC 2.5 gel group, the difference were not statistically significant ($p > 0.05$).

4. DISCUSSION

Box-Behnken design was used to optimize ACIN-loaded bilosomes. A total of 17 formulations were obtained by varying three formulation parameters (amount of lecithin, amount of sodium taurocholate and amount of cholesterol). The effect of these formulation variables on the formulation characteristics (zeta potential, vesicle size and EE%) was evaluated. As a result, the vesicle size, zeta potential, and EE% values for the optimum ACIN-loaded bilosomal formulation were 137.3 nm, -30.1 mV, and 84.5%, respectively.

Additionally, in this study, the gel formulations containing the optimum bilosomal formulation were prepared using Carbopol® Ultrez™ 10 or HPMC and evaluated *in vitro*. Firstly, the mechanical properties such as adhesiveness, cohesiveness, and hardness of the ACIN-loaded bilosomal gel formulations were evaluated using texture profile analysis.

Hardness, defined as the ability of the gel formulation to be removed from the container, was determined in our study (Table 2). A low hardness value indicates easy removal and easy application of the formulation, while on the other hand, it indicates that the retention time at the application site may be

shortened [27]. The molecular weight and concentration of the polymer have a significant effect on the hardness of the gel formulation. Sezer et al. reported that the hardness value of hydrogel increased significantly (5 times) due to the increase in the concentration of chitosan (from 1.5–2%) [28]. In another study, the hardness values of chitosan or polycarbophil gels increased four- and seven-fold, respectively, as the polymer concentration increased from 2–3% (for chitosan) and from 2–4% (for polycarbophil) [29]. In our study, an increase in the hardness value of ACIN-loaded bilosomal gel formulations was obtained with the increase in polymer concentration (especially for bilosomal Carbopol gel) (Table 2).

The higher cohesiveness, which is a significant parameter for determining the reconstruction ability of the gel after application, the better the structural recovery is generally observed. Thus, product performance at the administration site can be improved [27]. The increase in polymer concentration has not generally provided a significant increase in the cohesiveness of the bilosomal gel formulations (Table 2; $p > 0.05$).

For an effective treatment, gels should retain in the application area for the desired period of time. Therefore, the adhesiveness is another important parameter to be determined for gels. Sezer et al. reported that the concentration and molecular weight of the polymer used to prepare the gel affected the adhesiveness of the gel, and that adhesiveness increased as the polymer concentration and molecular weight increased [28]. In our study, there was an increase in the adhesiveness of ACIN-loaded bilosomal gel formulations with increasing polymer (Carbopol or HPMC) concentration (Table 2).

In addition, the pH values were determined the gel formulations. The pH of human skin, which is generally acidic, can vary greatly between 4.0 and 7.0 [30]. There is a general consensus that topical products should have an acidic pH and that their pH value should typically be in the range of 4–6 [31]. Therefore, the pH values of the bilosomal gel formulations prepared using HPMC were found to be more suitable for skin application (Table 6), and further studies were conducted on the bilosomal gel formulations prepared using HPMC.

Moreover, we assessed the dissolution of pure ACIN and the release of ACIN from the optimum ACIN-loaded bilosomal formulation or ACIN-loaded bilosomal gel formulations prepared using HPMC (2.5 or 5%) (Fig. 13**-A**). Approximately 90% of pure ACIN dissolved within 3 hours. However, approximately 15%, 32%, 46% and 55% of ACIN were released from the optimum ACIN-loaded bilosomal formulation in 0.5 h, 1 h, 3 h and 8 h, respectively (Fig. 13**-A**). The bilosomal formulation exhibited biphasic release profile with initial burst release (15%, 0.5 h; due to the release of ACIN on the surface of vesicle) followed by sustained release (55%, 8 h; due to the release of ACIN encapsulated within vesicle) (Fig. 13**-A**).

Ahmed ve ark. reported that bilosomal systems have biphasic release profile (initial burst release and later sustained release). They emphasized that lornoxicam on the surface of the bilosomal system is responsible for the initial burst phase, while the sustained phase is due to the high affinity of lornoxicam (lipophilic drug) to the bilosomal system [32]. Zafar et al. prepared luteolin-loaded bilosomal or pegylated bilosomal formulations and stated that both formulations exhibited biphasic release with initial fast release followed by sustained release, that the initial fast release could be due to the release

of luteolin from the surface of the vesicle, and that the slow release of luteolin from the formulations was due to cholesterol reducing membrane fluidity [33].

In addition, about 29%, 42%, 64% and 77% of ACIN were released from the ACIN-loaded bilosomal gel formulation prepared using HPMC (2.5%) in 0.5 h, 1 h, 3 h and 8 h, respectively (Fig. 13**-B**).

The high swellability of HPMC when in contact with water or biological fluid provides a faster drug release [34]. However, approximately 13%, 20%, 40% and 58% of ACIN were released from the ACIN-loaded bilosomal gel formulation prepared using HPMC (5 g) in 0.5 h, 1 h, 3 h and 8 h, respectively (Fig. 13**-B**). When the amount of HPMC was increased in the formulation, the release of ACIN from the bilosomal gel was slowed.

Pan et al. prepared HPMC hydrogels for topical application and reported that when the HPMC concentration was decreased from 13–12%, the drug release increased due to the decreased viscosity [35].

Moreover, MTT assay method was used to estimate the cell viability. In our study, the viability of cells was about 90% and above both optimum bilosome and bilosomal HPMC 2.5 gel formulations. Therefore, both prepared formulations are biocompatible.

Watroba et al. reported that according to ISO 10993, materials that provide over 70% cell viability are biocompatible, while those that reduce viability greater than 30%are cytotoxic [36].

CONCLUSION

In this study, a Box-Behnken design was used to prepare and optimize ACIN-loaded bilosome formulations. The optimized ACIN-loaded bilosome formulation had nano-size (137.3 nm), entrapment efficiency above 80% and negative zeta potential value (-30.1 mV; this value is sufficient for the physical stability of the colloidal dispersions). ACIN-loaded bilosomal gel formulations were also prepared using Carbopol or HPMC. Since the pH values (about 4) of ACIN-loaded bilosomal gel formulations prepared using HPMC (2.5 or 5%) were suitable for topical application, they were selected for further studies. The optimum ACIN-loaded bilosomes and ACIN-loaded bilosomal gel prepared using HPMC were able to sustain the ACIN release over eight days. For cytotoxicity assay, only ACIN-loaded bilosomal gel prepared using HPMC (2.5%) was evaluated in L929 cell line. This formulation did not cause a significant decrease in cell viability at different concentrations and was considered to be biocompatible.

AUTHORS' CONTRIBUTIONS

The authors confirm their contributions to the paper as follows: study conception and design by ED, MC and MSK; data collection, analysis and interpretation of the results by ED, TÇ, ABÖ and LB; manuscript drafting, revision, and/or correction by ED, MC and MSK; literature survey by ED, TÇ, ABÖ, LB, MC and MSK. All authors reviewed the results and approved the final version of the manuscript.

Declarations

AUTHORS' CONTRIBUTIONS

The authors confirm their contributions to the paper as follows: study conception and design by ED, MC and MSK; data collection, analysis and interpretation of the results by ED, TÇ, ABÖ and LB; manuscript drafting, revision, and/or correction by ED, MC and MSK; literature survey by ED, TÇ, ABÖ, LB, MC and MSK. All authors reviewed the results and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are not publicly available due to institutional policy restrictions but are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study did not involve any experiments on human or animal subjects; therefore, ethical approval from an institutional review board or animal ethics committee was not required.

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Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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Figures

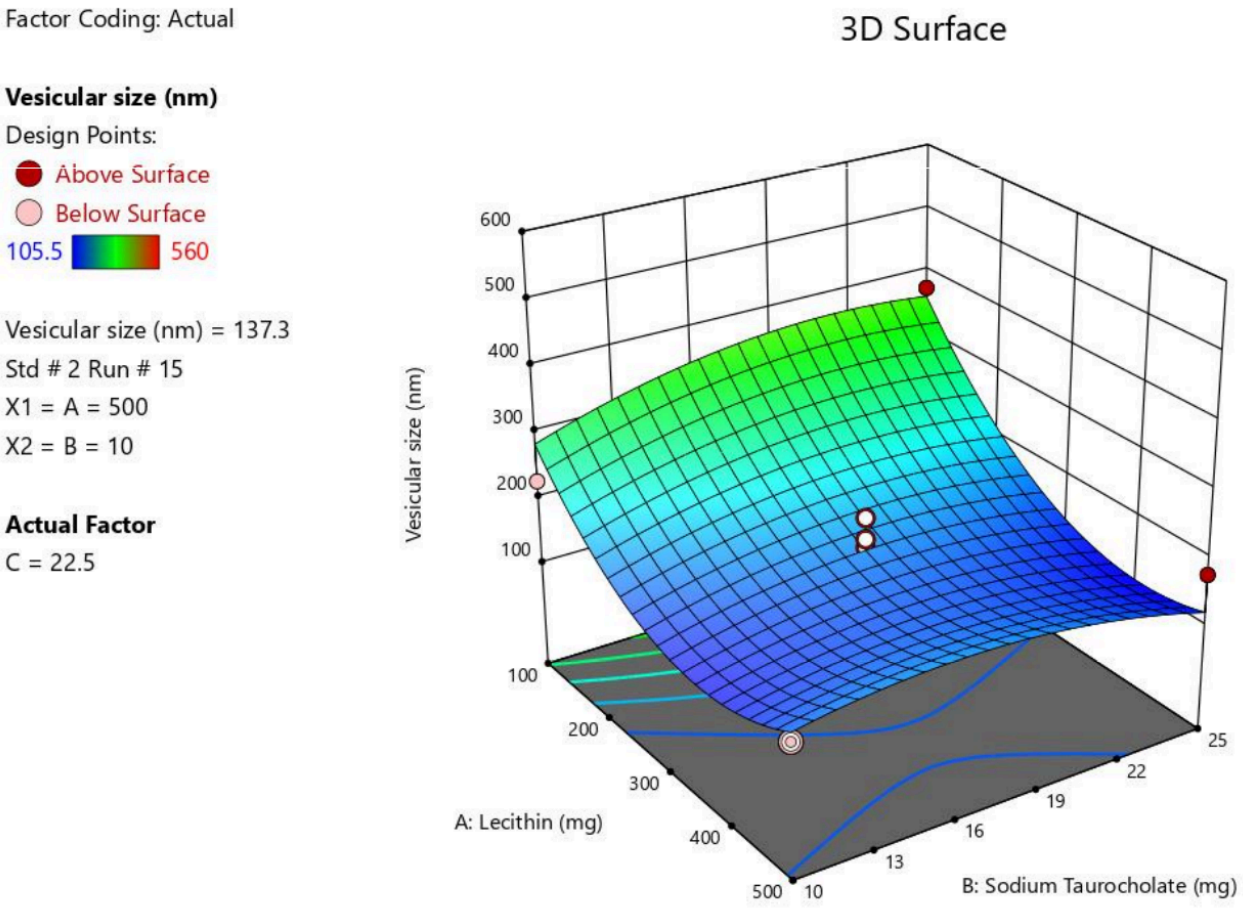


Figure 1

3D Surface showing the effect of amount of lecithin and sodium taurocholate on the vesicular size of ACIN-loaded bilosome formulations.

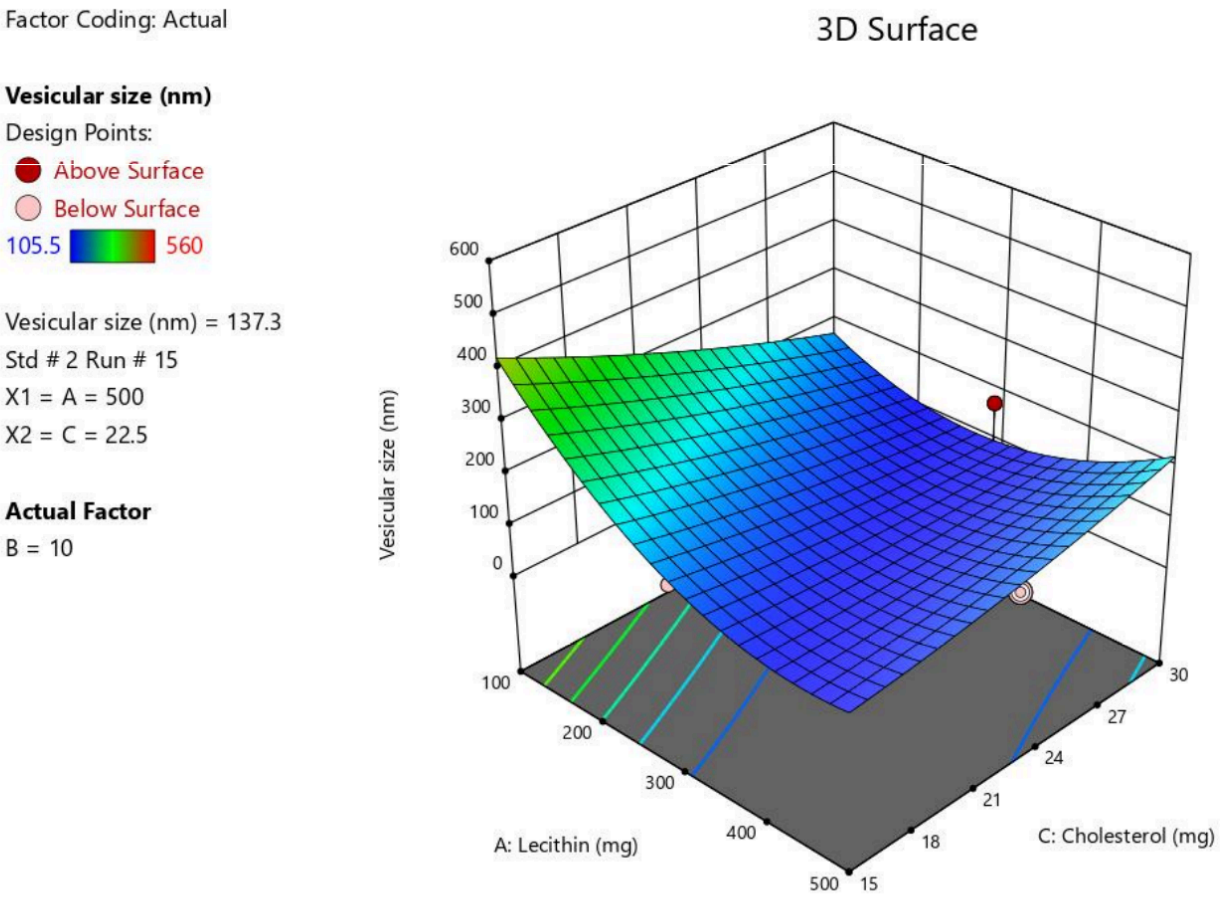


Figure 2

3D Surface showing the effect of amount lecithin and cholesterol on the vesicular size of ACIN-loaded bilosome formulations

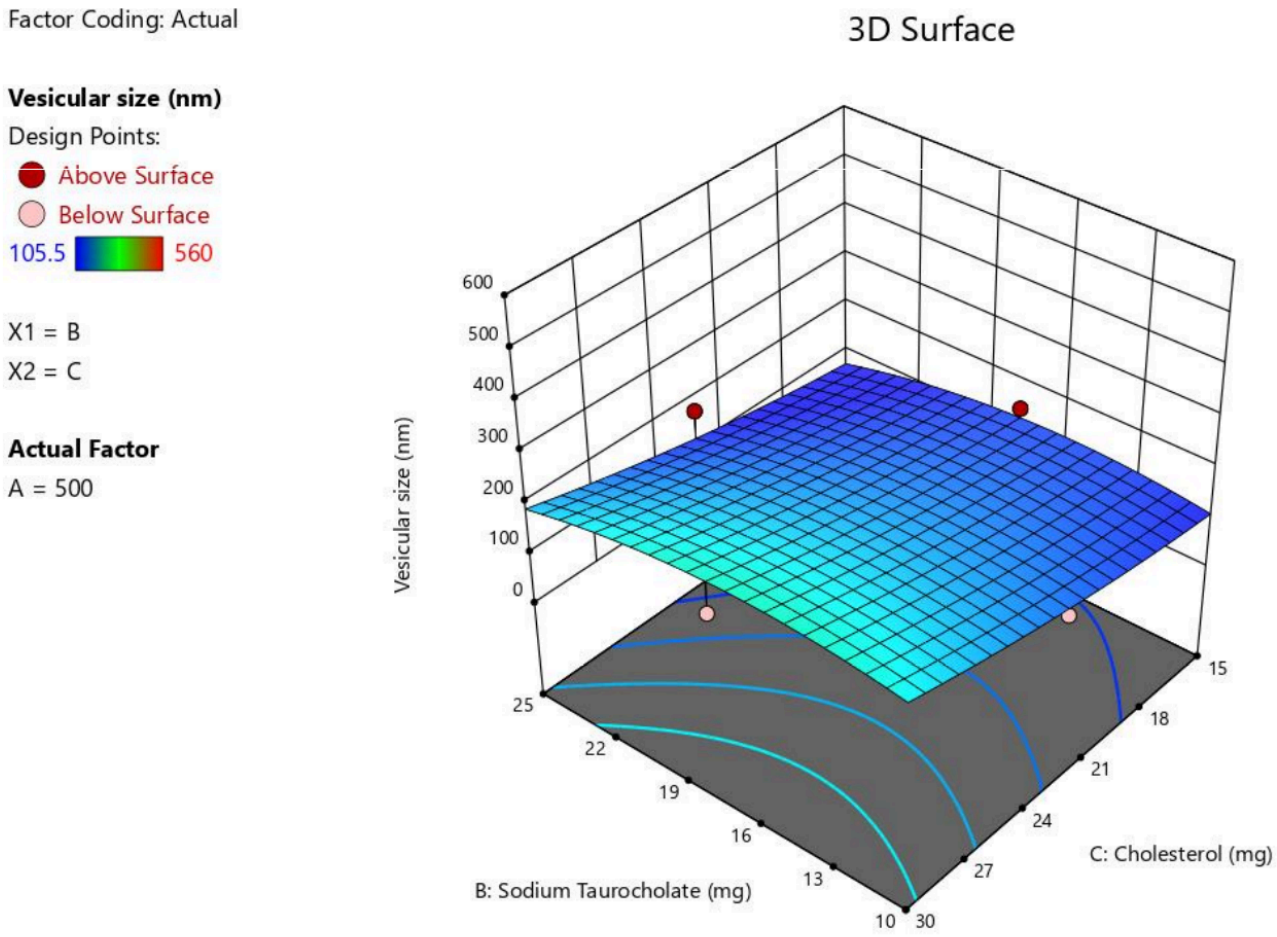


Figure 3

3D Surface showing the effect of amount of sodium taurocholate and cholesterol on the vesicular size of ACIN-loaded bilosome formulations

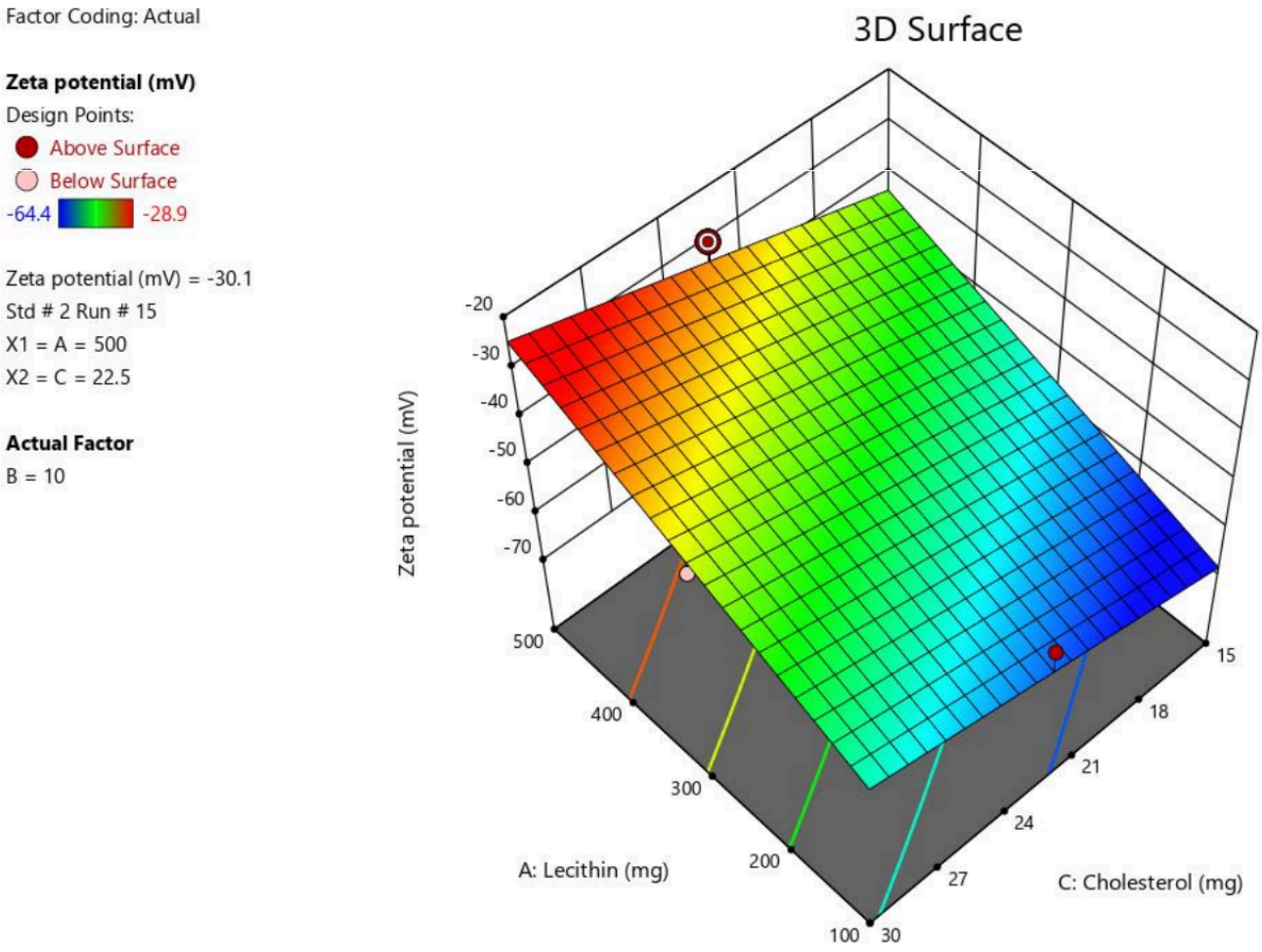


Figure 4

3D Surface showing the effect of amount of lecithin and cholesterol on zeta potential values of ACIN-loadedbilosome formulations

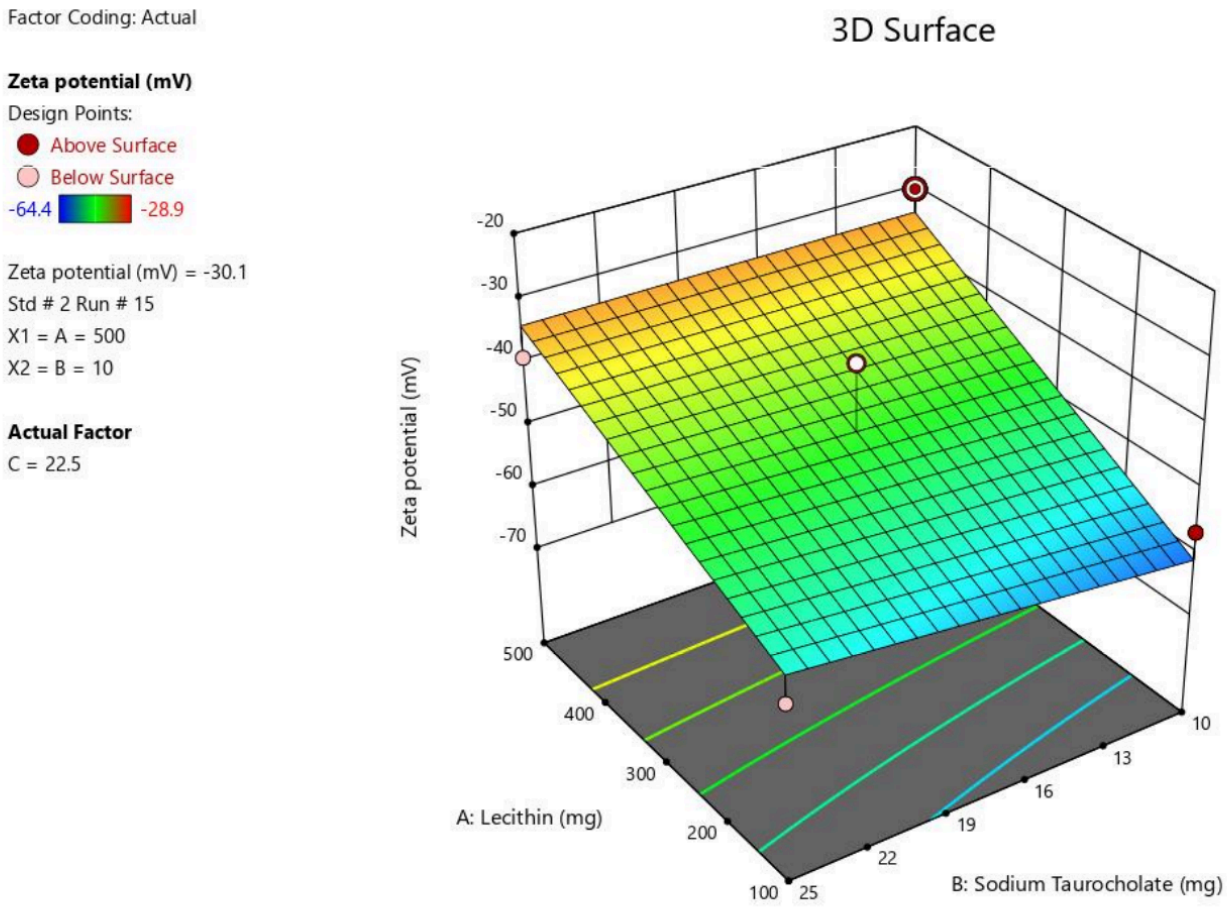


Figure 5

3D Surface showing the effect of amount of lecithin and sodium taurocholate on zeta potential values of ACIN-loaded bilosome formulations

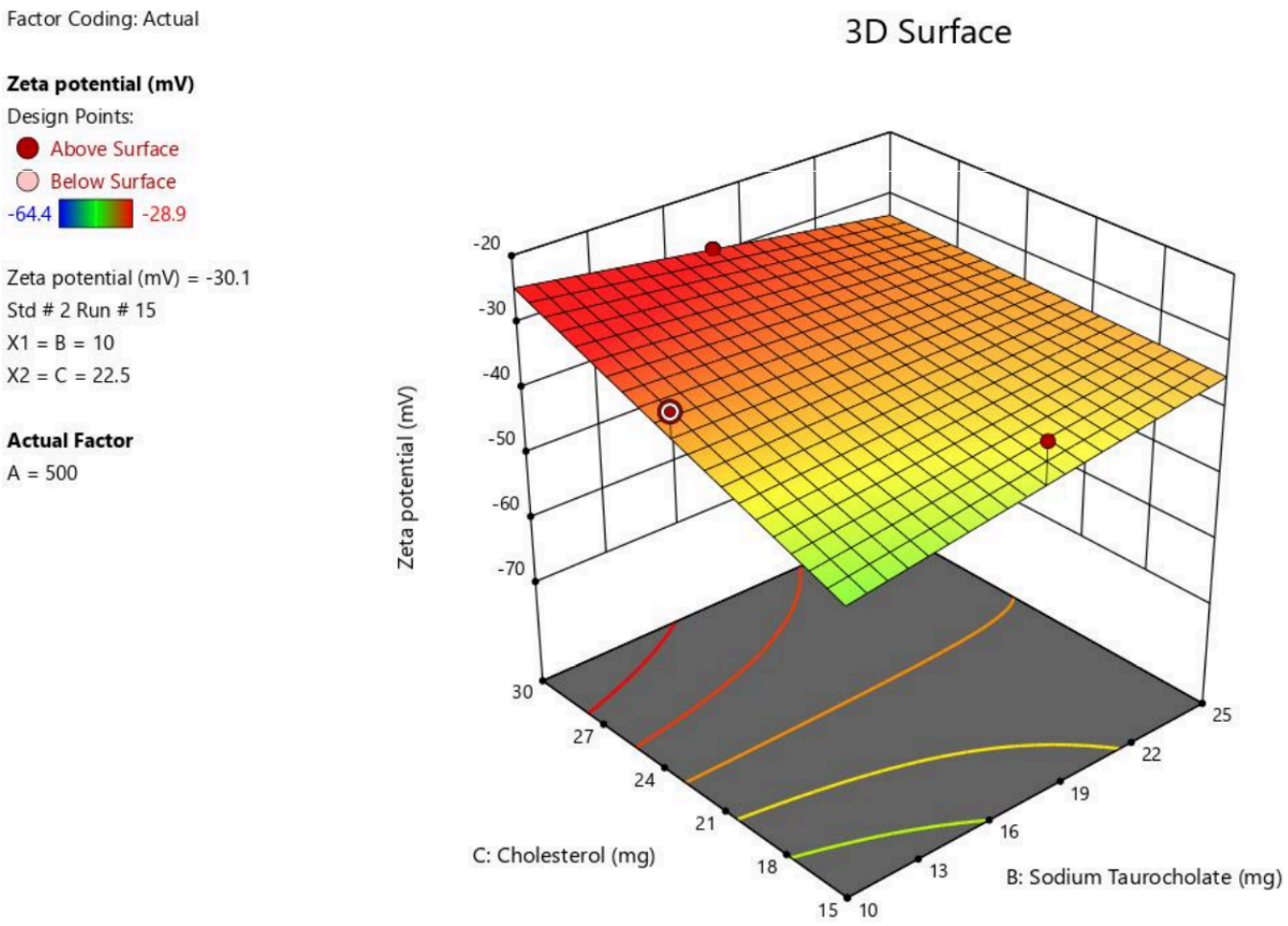


Figure 6

3D Surface showing the effect of amount of sodium taurocholate and cholesterol on zeta potential values of ACIN-loadedbilosome formulations

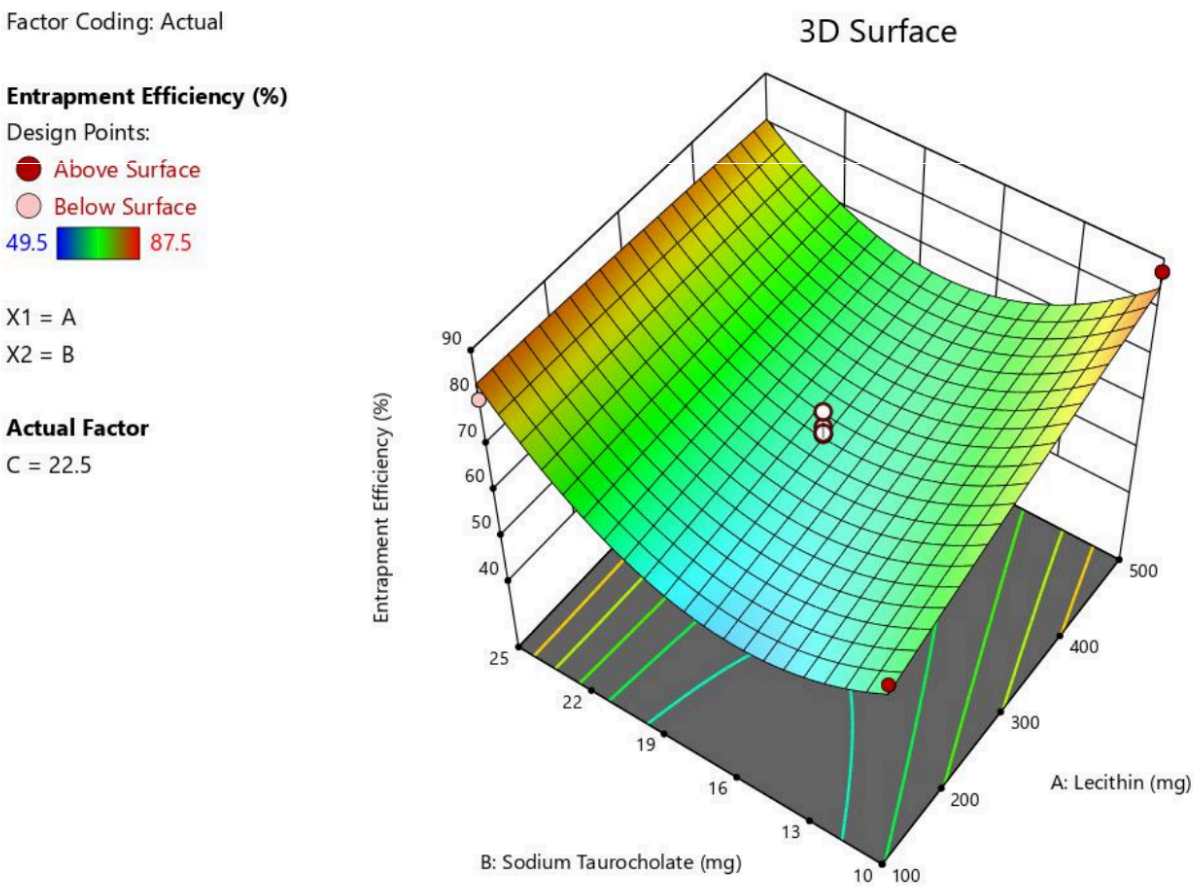


Figure 7

3D Surface showing the effects of amounts of sodium taurocholate and lecithin on EE%

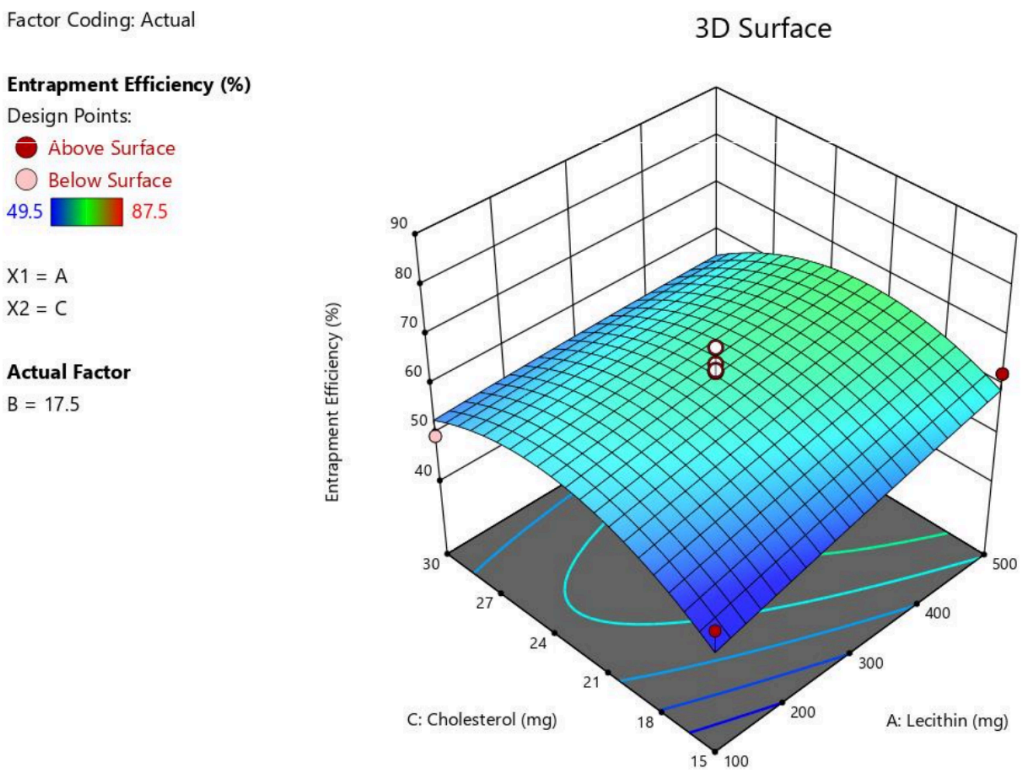


Figure 8

3D Surface showing the effects of amounts of lecithin and cholesterol on EE%

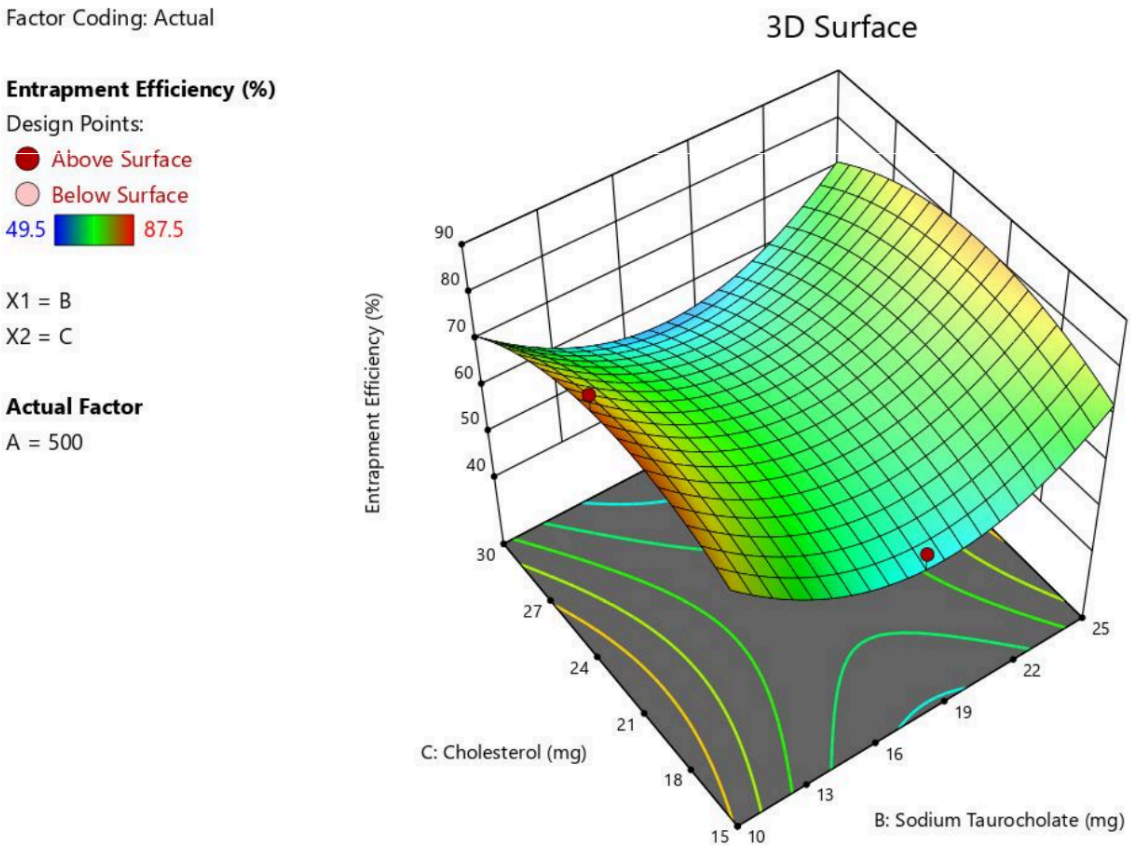


Figure 9

3D Surface showing the effects of amounts of sodium taurocholate and cholesterol on EE%.

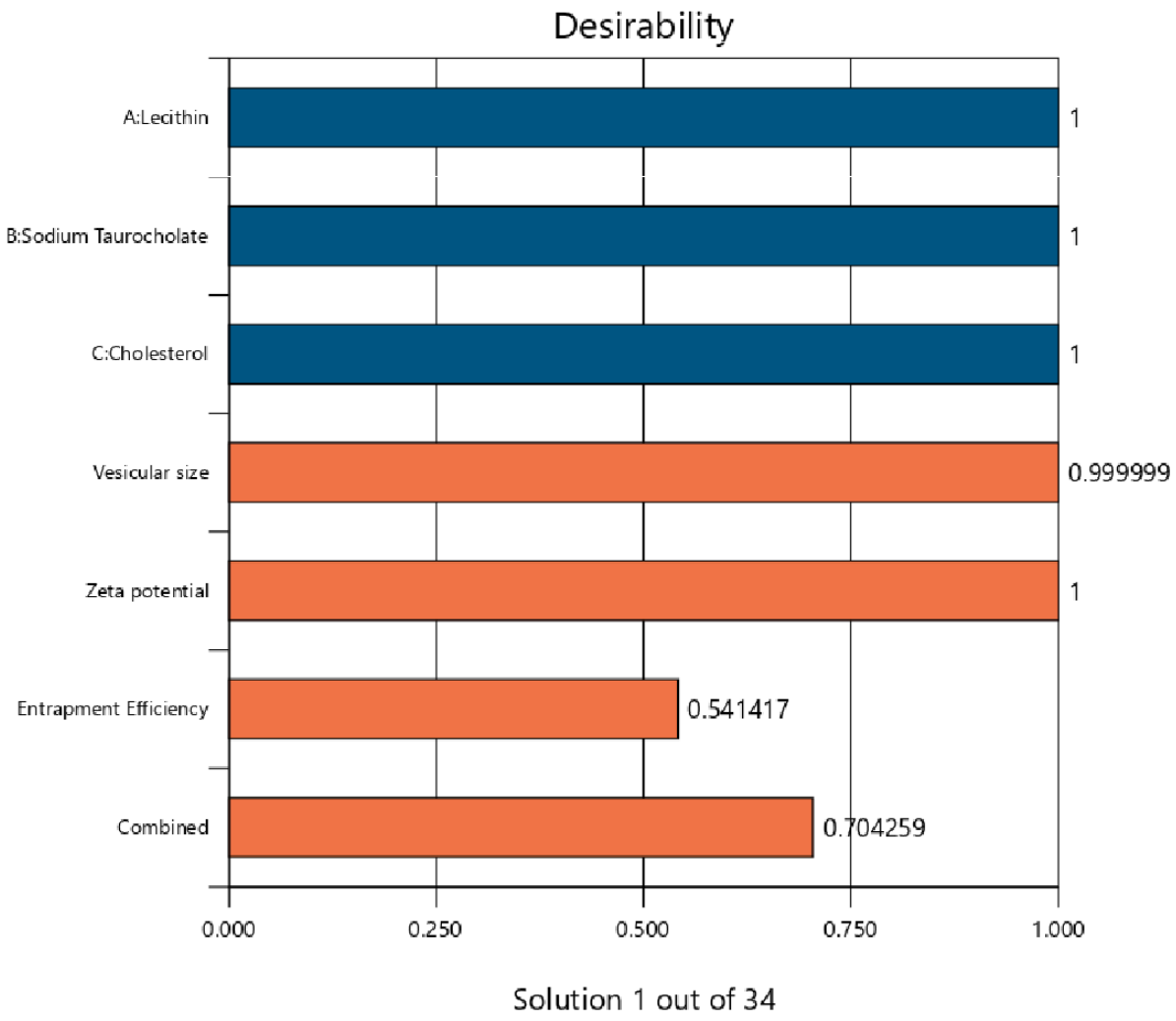


Figure 10

Desirability value of the responses: vesicular size, zeta potential and EE%

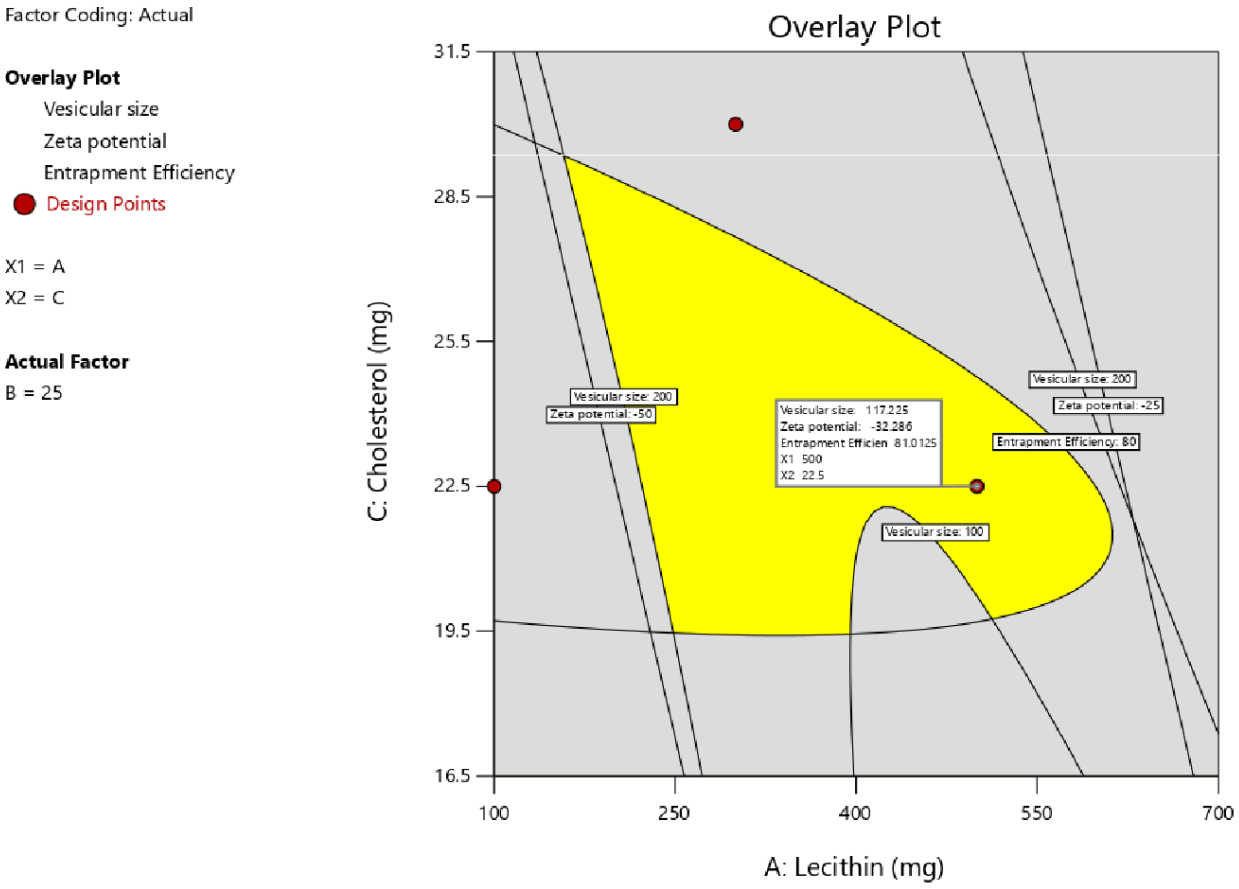


Figure 11

Overlay plot of the optimum levels of the responses: vesicular size, zeta potential and EE%

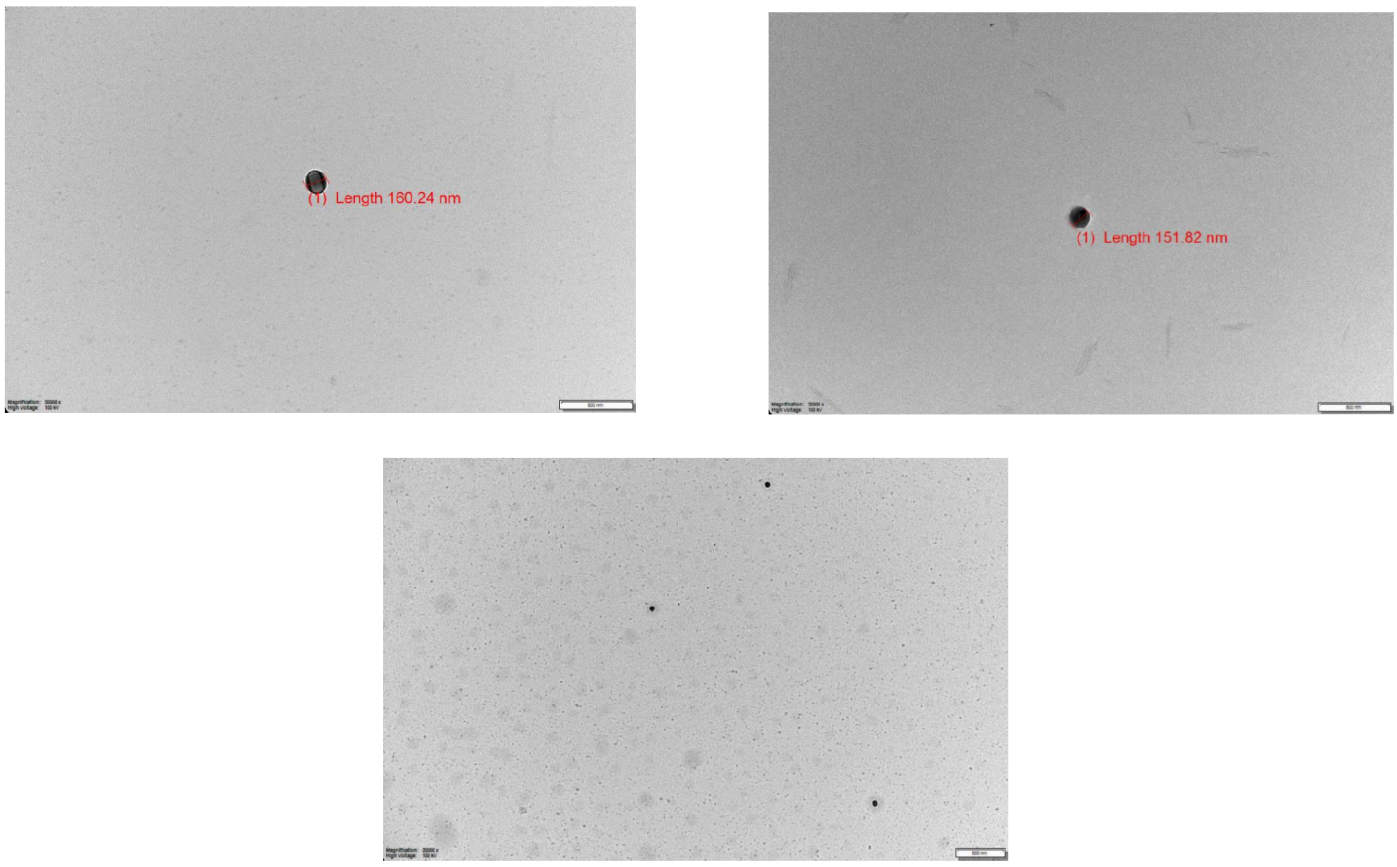


Figure 12

TEM images of optimum blank bilosomes (A) and ACIN-loaded bilosomes (Formulation no: 15) (B1 and B2)

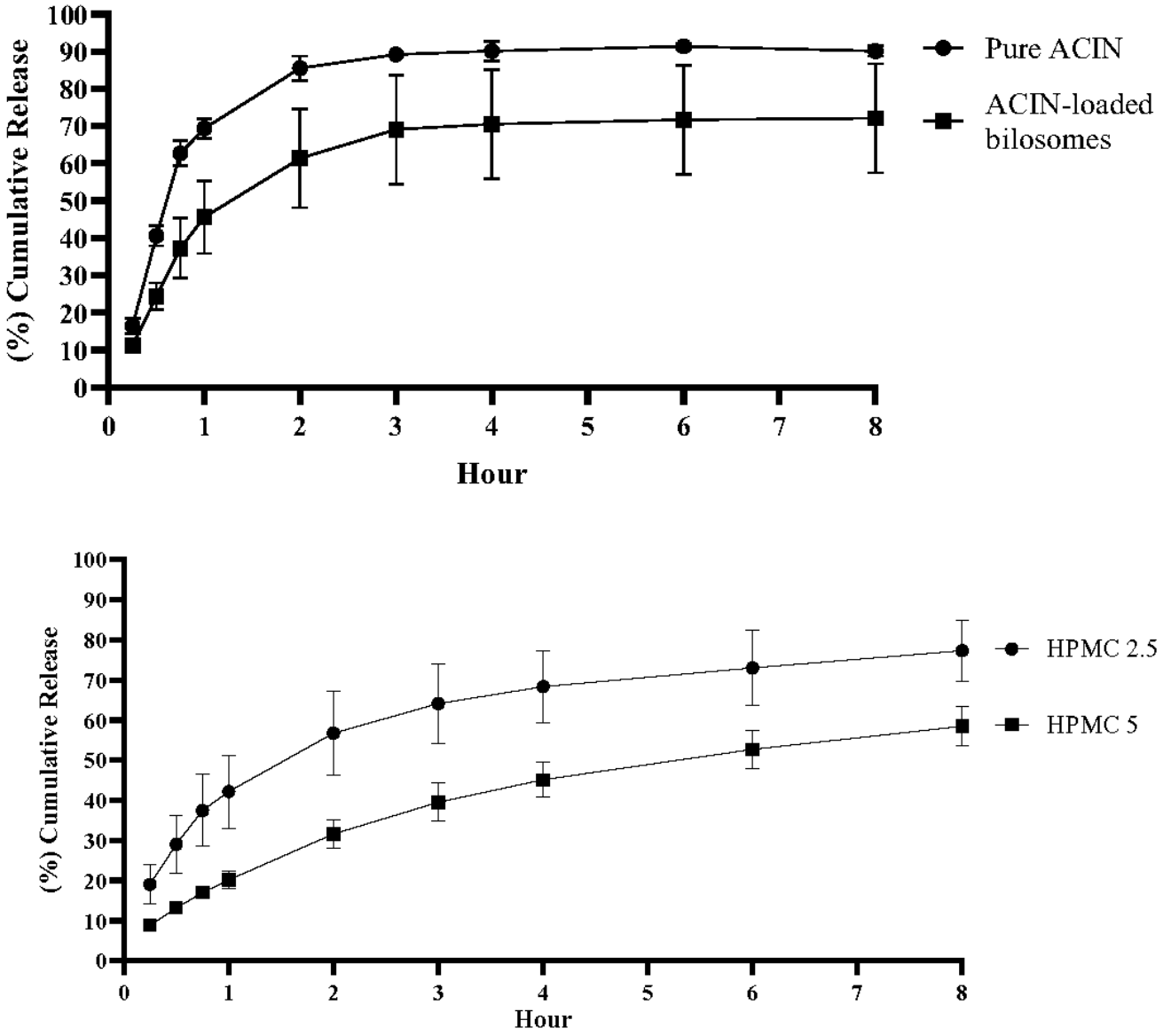


Figure 13

The dissolution profile for pure ACIN (A) and the in vitro release profiles of the optimum bilosome formulation containing ACIN (A) and ACIN-loaded bilosomal gel formulations [HPMC 2.5 and HPMC 5: ACIN-loaded bilosomal gel formulations prepared using HPMC (2.5 or 5%)] (B)

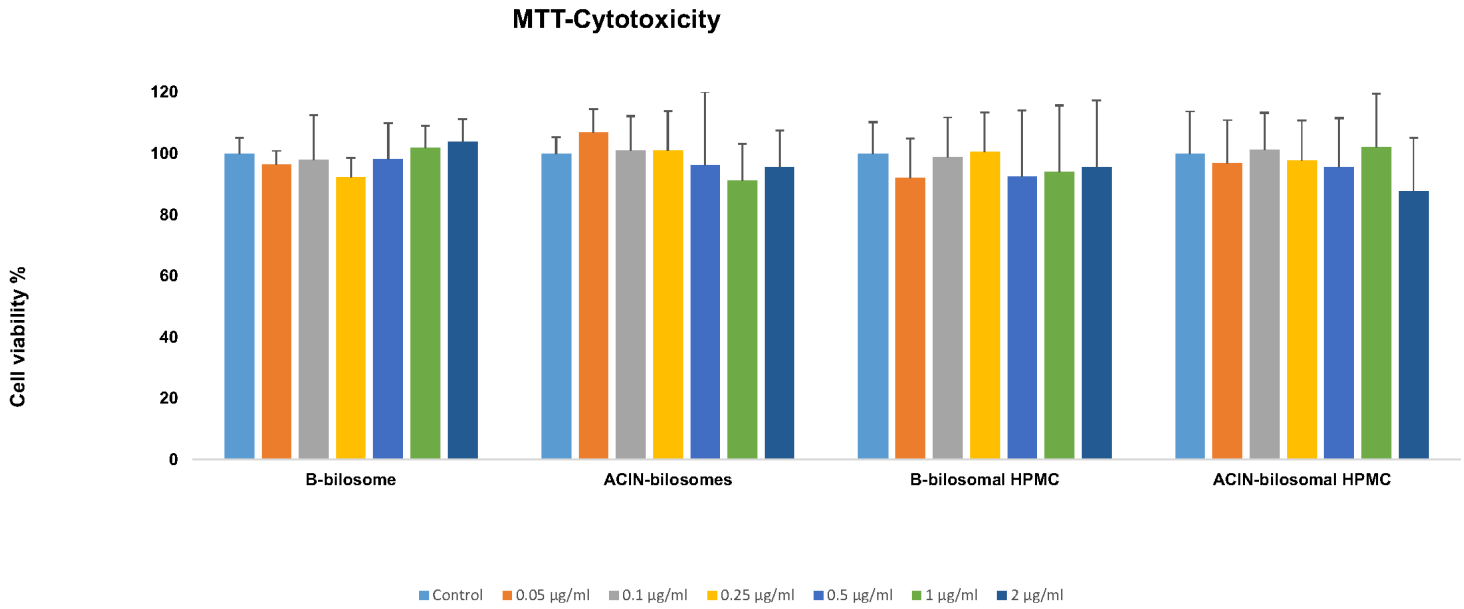


Figure 14

The viability of cells treated with the bilosomes and bilosomal HPMC 2.5 gel formulations (after 24 h incubation) (**Control:** Untreated cells; **B-bilosome:** Blank bilosomes; **B-bilosomal HPMC:**Blank bilosomal gel prepared using HPMC; **ACIN-bilosomes:** ACIN-loaded bilosomes; **ACIN-bilosomal HPMC:** ACIN-loadedbilosomal gel prepared using HPMC.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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