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Preparation and Optimization of Vancomycin hydrochloride Encapsulated Multivesicular Liposomes for Sustained Locoregional Delivery

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Abstract:

Introduction: Osteomyelitis is a destructive inflammatory condition of the bone that is usually caused by a wide range of microorganisms especially *Staphylococcus aureus*. Considering the downsides of systemic antibiotic therapies as well as conventional local drug delivery systems such as using polymethylmethacrylate, this study aimed to develop, characterize and optimize vancomycin hydrochloride loaded multivesicular liposomes (MVLs) as a proper therapeutic option for the treatment of osteomyelitis.
Methods and Results: A 2^3 full factorial design technique was applied to determine the effects of three variables (lipid to drug ratio, triolein content and cholesterol to phospholipid ratio) on the encapsulation efficiency and release profile of vancomycin hydrochloride loaded MVLs to optimize the final formulation. Further characterization was performed on the optimized formula by evaluating the morphology, size and storage stability. The average drug encapsulation efficiency and the mean diameter of the optimized formulation was 54.7 ± 0.3% and 9.019 ± 0.26 µm, respectively with a span value of 0.188. Additionally, the spherical and multivesicular nature of MVLs was visible using optical microscopy (x400). The optimized formula showed an in vitro sustained release characteristic with proper stability and insignificant change in size, morphology and EE% for 30 days at 4°C.
Conclusion: This study suggests that vancomycin hydrochloride loaded MVLs might have the potential to be used in the treatment of chronic osteomyelitis as a biocompatible drug carrier with a high antibiotic entrapment capacity as well as controlled drug release.

Keywords: Vancomycin hydrochloride; Multivesicular liposomes; Osteomyelitis; Sustained release; Factorial design.

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1. Introduction

Osteomyelitis is a destructive inflammatory condition of the bone that is usually caused by an invasive infection and is identified by pathological changes in bone regeneration. Nowadays, the incidence of this disease is increasing and can involve all ages and each bone, and become a chronic disease and cause persistent morbidity [1]. Treatment is especially challenging when a complex multidrug-resistant bacterial biofilm is developed. Bacteria in the biofilm remain in a low metabolic stage and could cause persistent infection due to increased resistance to antibiotics. Furthermore, some host factors, make treatment of the disease even more challenging such as the formation of a "sequestrum" at the site of infection, which prevents penetration of antimicrobial agents into the infected site. Although this infection can be caused by a wide range of microorganisms,

Staphylococcus aureus and *Staphylococcus epidermidis* are the most common causes of the disease respectively, which are responsible for more than 50% of cases of osteomyelitis [2].

The conventional treatment methods of osteomyelitis consist of the removal of necrotic tissue, rinsing the infected site with a proper antiseptic, and administrating a high dose of prolonged parenteral (at least 4 to 8 weeks) antibiotic treatment. These systemic therapeutic methods have some serious disadvantages such as nephrotoxicity, ototoxicity, and gastrointestinal side effects, as well as bacterial resistance in case of insufficient doses of antibiotics or incomplete treatment [3,4].

Although oral antibiotics administration has some upsides including ease of use, cost decrease, and shorter period of hospitalization, it also has some downsides such as the unpredictable therapeutic efficiency, limitation of drug type and prescribed dosage, treatment duration, and improper drug penetration to the infected area [2].

Given all these limitations and deficiencies, local antibiotic therapy offers several advantages such as providing high local concentration with lower systemic effect over parenteral and oral drug administration [2,5].

In recent years, non-biodegradable drug carriers named PMMA (polymethylmethacrylate) have been fostered. Commercial PMMAs are available in the form of beads (Septopal ®) and Cement.[2] Although usage of PMMAs has rectified the drawbacks of intravenous antibiotics to some extent, still it has some important downsides which cause several issues for patients. Some of these drawbacks are mentioned below:

1. Since PMMAs are non-biodegradable, a second surgery to remove the carrier is inevitable which imposes more pain, cost and longer treatment duration [2,4,6]
2. Many studies declare that the use of PMMAs could delay the bone restoration process [4].
3. In the course of the polymerization process, due to the liberation of excessive amounts of heat, antibiotics may be neutralized [2].
4. In some cases, either antibiotic release is not entirely accomplished or is completed very slowly, the use of PMMAs could cause antibiotic resistance [4].
5. PMMAs placement and removal processes increase the risk of secondary infection considerably [7].
6. PMMAs could become an appropriate surface for biofilm formation [6].

Based on the mentioned issues, in recent years, the design of biodegradable local drug delivery systems is a prevalent case study among many researchers. Some studies in the biomaterial field, have performed biodegradable scaffolds designs to find alternatives for current PMMAs. Bioceramics, bioactive glasses, polymer scaffolds and composites are among these materials which have their own advantages and drawbacks. The common upside of these scaffolds compared to PMMAs is absence of the second surgery for removal of the carrier, but the main disadvantage of these methods is still the necessity of the primary surgery for placement of these carriers [4].

MVLs, also known as Depofoams, are lipid-based and sustained-release carriers that contain multiple nonconcentric aqueous chambers. MVLs have many advantages over other classic types of liposomes namely having a significantly slower drug release rate and higher storage stability. Compared to polymeric encapsulation systems, Depofoams are both biodegradable and biocompatible as they are derived from natural lipids. The spacious internal aquatic chambers in MVLs allow a highly efficient loading of hydrophilic drugs along with a controlled and prolonged release profile. Their close-packed nonconcentric structure allows them to rearrange their vesicles internally and also is responsible for MVLs stability and drug sustainability [10,11,12].

Since the 1950s ,vancomycin hydrochloride (Van. H) has been used against gram positive bacteria such as *Staphylococcus aureus*. Nowadays, this glycopeptide antibiotic is still widely used as one of the most effective treatment options for bone infections. *S. aureus* is one of the most pathogenic origin of osteomyelitis and Van. H is one of the few available antibiotics to treat infections caused by *S. aureus* and methicillin-resistant Staphylococcus aureus (MRSA) [2,8,9].

Considering the above-mentioned advantages of local delivery of antibiotics and effectiveness of Van. H for treatment of osteomyelitis, as well as benefits of MVLs as a biocompatible carrier with a high ability of sustained drug release, this study aimed to develop and characterize Van. H loaded MVLs as a proper therapeutic option for treatment of osteomyelitis. A 2^3 full factorial design technique was applied to determine the effects of three variables (lipid to drug ratio (L/D), triolein content (To%) and cholesterol to phospholipid ratio (Chol/PL)) on the encapsulation efficiency and release profile of the MVLs to optimize the final formulation.

2. Materials & Methods

2.1. Materials

Van.H was purchased from Jaber Ebne Hayyan Pharmaceutical Co. (Tehran, IRAN). Amphipathic lipid, purified egg phosphatidylcholine r (EPC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Negatively charged lipid (dicetyl phosphate or DCP), cholesterol, neutral lipid (triolein) and free base L-Lysine were purchased from Sigma–Aldrich (USA). Chloroform and methanol were supplied by Merck (Germany). Dextrose 5% was obtained from Iranian parenteral and pharmaceutical Co. (Tehran, IRAN).

2.2. Preparation of multivesicular liposomes

Van H-MVL was prepared by double emulsion (w/o/w) method described in previous reports [13,14] Figure 1 demonstrates the schematics process of MVLs preparation. First, a water in oil emulsion was prepared by mixing 1 ml aqueous phase containing 6 mg/ml Van. H and 5% Dextrose, and 1 ml oil phase containing chloroform solution with amphipathic lipid (Egg PC), cholesterol, triolein and DCP using 15 min vortex (12,000 rpm). Subsequently, the second aqueous solution containing 40 mmol/L lysine was mixed with the primary emulsion for 1 min (10,000 rpm). Then the chloroform was removed by using a rotary evaporation. The resulting MVLs were isolated by centrifugation at 7500 rpm and washed twice with normal saline 0.9% to remove unentrapped Van. H, then resuspended in a L-lysin solution and stored at 4°C.

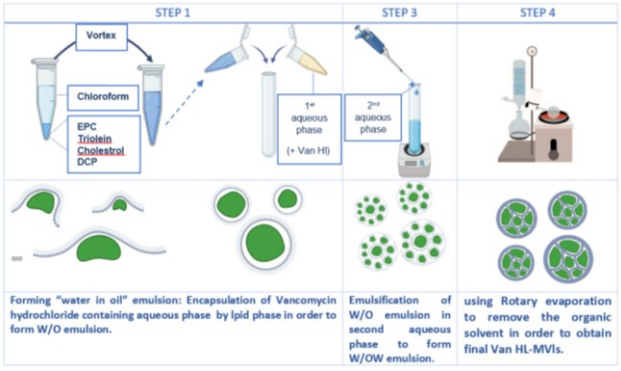


Figure 1. Schematic presentation of the preparation process of Van. H loaded MVLs.

2.3. Effect of variables

A two-level factorial design using Design-Expert® software (Version 12, Stat-Ease Inc., Minneapolis, MN, USA) was applied to study the effects of three different variables including L/D, To% and Chol/PL on the encapsulation efficiency and release profile. Each independent variable was set at three (low, midpoint and high) levels (Table 1). The values of each variable was considered according to preliminary studies. The Encapsulation efficiency, percentage of drug released after 24 h (DR24h%) and percentage of drug released after 48 h (DR48h%) were chosen to be dependent variables. The effects of the three independent variables were studied through 12 experiments. Besides, three center points were added to estimate the experimental errors. All batches were prepared randomly in duplicate pursuant to run orders and analyzed triplicate for entrapment efficiency (EE) and percent drug release. Each response coefficient and significance of the effects of variables were investigated using ANOVA, Pareto chart and Perturbation plot. Two-dimensional contour plots and three-dimensional response surface plots were used to study the combined effects of independent variables on the selected response. Statistical comparisons were evaluated by ANOVA with a significance level of $P < 0.05$.

Table 1. Investigated independent formulation variables and their levels used in a 2^3 full factorial design.

Independent formulation variables	Levels		
	Low (-1)	Midpoint (0)	High (+1)
A: Chol/ PL molar ratio	1.0	2.0	3.0
B: L/D molar ratio	20	30	40
C: To%	10	15	20

Abbreviations: Chol/PL, cholesterol to phospholipid ratio; L/D, lipid to drug ratio, To%: triolein percentage.

2.4. MVL characterization

2.4.1. MVL morphology, size and size distribution

The morphology of vesicles was determined using an inverted microscope (×400) connected to a digital camera (Optika, Italy). Particle size and size distribution were measured by a Mastersizer 2000 (Malvern Instruments, UK).

2.4.2. MVL Encapsulation Efficiency (EE%)

To determine Van.H content in the MVL formulations, a sample of each formulation (100 µl) was lysed with methanol (900 µl) and further vortexed for 5 min.
The UV spectrophotometry (UV-mini 1240, Shimadzu, Japan) method at 281 nm was used for quantitative estimation of Van H entrapped in MVLs. All the measurements were repeated three times and EE% was calculated using under-mentioned formula [15]:

$$\textbf{Encapsulation efficiency (\%)} = \frac{Actual\ drug\ content}{Theoretical\ drug\ content} \times 100$$

2.4.3. In vitro release

The in vitro release study of Van. H-MVL was determined at 37 °C using dynamic dialysis technique. [11] All formulations considering their specific encapsulation efficiency were used for this analysis. Briefly, 500 µl of each formulation was kept in a dialysis bag with a molecular weight cut-off of 14 kDa, and the system was immersed in 15 ml of pH 7.4 phosphate buffer saline (PBS) solution. The medium was shaken at 37±0.5 °C and 50 rpm. At regular time intervals (1, 2, 4, 8, 12, and then every 24 h), 500 µl of the release medium was taken out and was replaced with same volume of fresh medium in order to maintain the sink condition. Samples were collected for up to 7 days. The percentage of drug

released was calculated using the UV spectrophotometry according to the standard curve of Van. H at λmax=281 nm. The mean calculated values were determined from 3 replicates.

2.4.4. Storage Stability

The optimized Van H-MVLs were stored at 4°C for 30 days. The stability of MVLs was evaluated by measuring some stability indicators including size, morphology, and EE% at the determined time points.

2.5. Statistical analysis

All tests were performed in triplicate and results were represented as mean ± standard deviation (SD). Statistical comparisons were carried out using ANOVA and differences were considered statistically significant for $p < 0.05$.

3. Results and Discussion

3.1. Analysis of 23 factorial design

Experimental design has been recognized as a helpful and practical tool for formulation development and optimization as it represents an efficient approach to evaluate all formulation factors systematically in a timely manner [16].

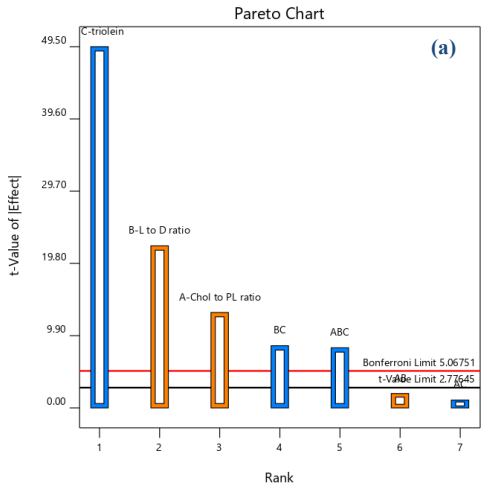
As lipid concentration and composition could have a direct effect on MVL efficacy (EE% and release rate), we investigated different properties of the MVLs prepared at varying Chol/PL molar ratios, L/D molar ratios and To%. The ranges of these parameters were firstly determined based on preformulating studies. The effect of these three variables on three response parameters including EE%, 24 hours release rate (DR24h%) and 48 hours release rate (DR48h%) was designed via a 2^3 full factorial design (Table 2).

Table 2. The independent variables and observed responses, of vancomycin hydrochloride loaded MVLs formulations according to a full 2^3 factorial design. (mean ± SD, n=3).

Run	Independent variables			Responses		
	Factor A (Chol/PL) *	Factor B (L/D) *	Factor C (To%) *	EE%*	DR24h%	DR48h%
1	2.00	30.00	15.00	37.2 ± 0.3	72.9 ± 0.7	86.2 ± 0.6
2	1.00	40.00	20.00	30.2 ± 0.2	53.3 ± 0.6	68.4 ± 0.3
3	2.00	30.00	15.00	36.8 ± 0.5	71.2 ± 0.3	84.9 ± 0.1
4	3.00	40.00	20.00	31.87 ± 0.3	67.8 ± 0.5	79.3 ± 0.6
5	3.00	20.00	20.00	30.16 ± 0.4	84.5 ± 0.8	94.6 ± 0.2
6	2.00	30.00	15.00	37.01 ± 0.1	73.8 ± 0.5	87.0 ± 0.4
7	1.00	40.00	10.00	47.65 ± 0.5	66.7 ± 0.5	81.3 ± 0.8
8	3.00	20.00	10.00	40.32 ± 0.7	77.6 ± 0.6	92.6 ± 0.1
9	3.00	40.00	10.00	54.7 ± 0.3	64.6 ± 0.8	79.6 ± 0.5
10	2.00	30.00	15.00	36.65 ± 0.2	71.8 ± 0.2	84.2 ± 0.3
11	1.00	20.00	20.00	24.87 ± 0.4	69.4 ± 0.4	82.2 ± 0.6
12	1.00	20.00	10.00	39.18 ± 0.1	78.3 ± 0.6	91.6 ± 0.4

***Abbreviations: Chol/Pl: Chol to phospholipid molar ratio, L/D: lipid to drug molar ratio, To: Triolein, EE: encapsulation efficiency**

Following the responses calculation (EE%, DR24h%, DR48h%), ANOVA test and Pareto charts were applied to examine the statistical significance of the parameters and the insignificant ones were excluded from the study (Figure 2). Through evaluation of the Pareto chart which represents the effects of variables by displaying two significance thresholds (t-value and Bonferroni limit). The effects situated above the Bonferroni line are indicated statistically significant, and those located between the Bonferroni and t-value limit lines are demonstrated to be possibly significant, while those under the t-value limit are statistically insignificant and should be eliminated from the analysis [17].



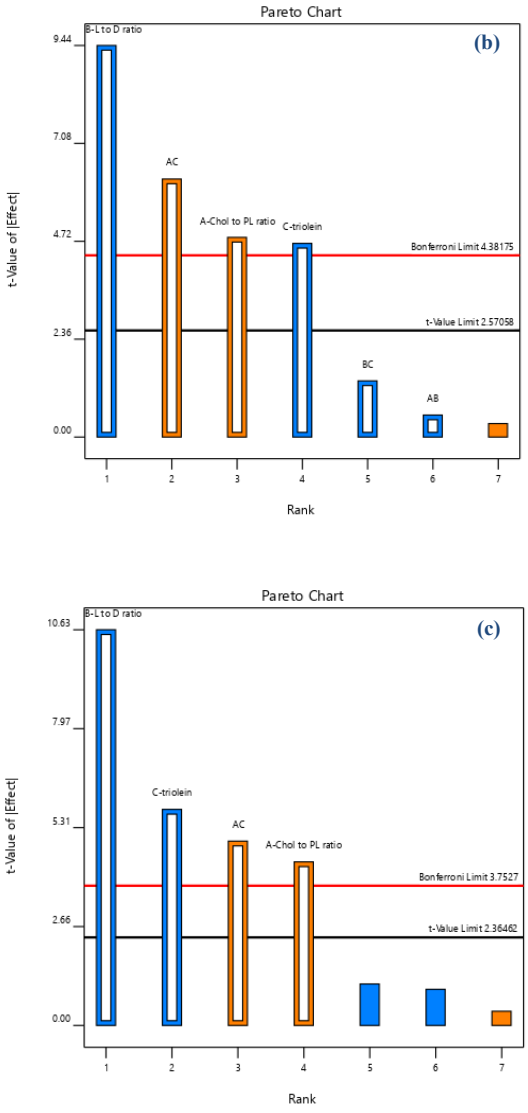


Figure 2. Pareto chart of the analyzed effects for (a) EE%, (b) DR24h% and (c) DR48h%

3.2. Effects of variables on encapsulation efficiency:

As seen in Table 2, MVLs were obtained with EE% in the range of 24.87% to 54.7%. To describe the effect of three formulation variables on EE%, multiple linear regression analysis (MLRA) was applied and the suggested polynomial model was then simplified by removing non-significant terms ($P > 0.05$). The obtained model is expressed as the following equation:

1. $Y1\ (\%EE) = +36.28 + 0.9462\ A + 3.59\ B - 8.02\ C + 0.1425\ AB - 0.0775\ AC - 1.37875\ BC - 0.5963\ ABC$

As mentioned earlier, the A, B, C, terms stand for the Chol/PL molar ratio, L/D molar ratio, and the To%, respectively. As shown in in the ANOVA analysis, the model is significant (F-value of 602.44 ($P < 0.0001$)) and there is only 0.01% chance that such a large F-value could arise due to noise. Besides, the lack of fit with the F-value of 9.08 (P-value of 0.0571) is statistically insignificant which means our model fits the actual response behavior well. Furthermore, the difference between the predicted R-squared (0.8624) and the adjusted R-squared (0.9974) is less than 0.2, indicating a reasonable agreement between the two values. Adequate Precision measures the signal to noise ratio and a ratio more than four is desirable. Consequently, our ratio of 89.181 specifies an adequate signal and this model can be applied to navigate the design space. The Pareto plot (Fig 2.a) and the equation demonstrate that the factor C (To%) has a remarkable negative effect on the EE% with the F-value of 2449.80 ($P < 0.0001$) while there is a positive correlation between both A (Chol/PL) and B (L/D) variables and Van.H entrapment with F-value of 170.73 ($p=0.0002$) and 492.86 ($p < 0.0001$), respectively.

In the MVL structure, employing neutral lipids such as triolein is critical for the stabilizing of multivesicular liposomes structure [18]. The results of microscopic and NMR evaluations have shown that triolein, not only acts as a stabilizing agent and hydrophobic filler in the spaces between vesicles junctions, but also present in the encapsulated aqueous phase as dispersion of oil droplets [13].

According to previous reports, the optimal concentration of triolein has a profound effect on the percentage of EE [18]. Triolein, in a proper range, increase the stability of the MVL structure and therefore impose an additive effect on the EE. However, it would reduce the encapsulation efficiency in higher amounts. Given that the Van.H is a large hydrophilic molecule with a molecular weight of 1485.7 g/mol, [19] it is expected to be placed in the internal aqueous space of the liposome. It seems that using higher proportion of triolein, by occupying the internal aqueous space, limits the entrapment capacity of this large molecule and therefore has a reducing effect on EE%.

A positive correlation was shown between Chol/PL ratio (A variable) and Van. H entrapment. The increase in EE% with increasing the Chol/PL ratio can be due to the reason: that increasing the cholesterol as a membrane fluidity buffer can lead to increased membrane stability, reduced drug leakage throughout the MVL preparation process and eventually enhancing EE%.

The positive impact of L/D ratio (variable B) on the EE may be explained by the fact that due to the special structure of multivesicular liposomes, increasing the concentration of lipid can lead to growth in the number of vesicles per milliliter and subsequently the final volume for drug encapsulation per milliliter, and drug carrying potential is increased [20].

The perturbation plot is a useful representative tool to compare the effects of all the factors at a particular point in the design space and helps to find factors that affect the response the most. The response is graphed by changing only one factor over its range. while other variables are maintained constant. The curvature or steep slope in this graph determines the response sensitivity to that parameter [21,22]. As demonstrated in Figure 3, a steep slope for factor C (To%) and a slight slope for factor A (Chol/PL) indicates that the To% is a significant factor for controlling the EE%. Given that the slope of the graph is negative for this factor, it has a remarkable decreasing effect on the selected response (EE%).

The combined effects of independent variables on selected response were further studied by Response surface methodology including two-dimensional contour plots and three-dimensional response surface plots. These plots were drawn by maintaining one variable constant at the center point while changing the other two variables within the experimental range. The 3D response surface plots are very helpful for studying both the main and interaction effects of the independent factors. The 2D contour plots give visual clues on the values of the response in order to optimize the response function [23,24].

As illustrated in Figure 4 (a and b) by keeping the factor A constant at the highest value (Chol/PL=3), the combined effects of the other two factors on the response were investigated. The results showed that increasing the L/D ratio to its highest level (40) while reducing the To% to 10%, would cause the highest EE (50% and more). As shown in Figure 5 (a and b) by maintaining factor C constant at the lowest value, the maximum value of EE is obtained by increasing the other two factors. According to Figure 6 (a and b), by keeping the factor B constant at the value of 40, EE% will increase as factor C decreases and factor A increases. The findings of 3D response surface plots and contour plots analysis confirm the results of the Pareto chart.

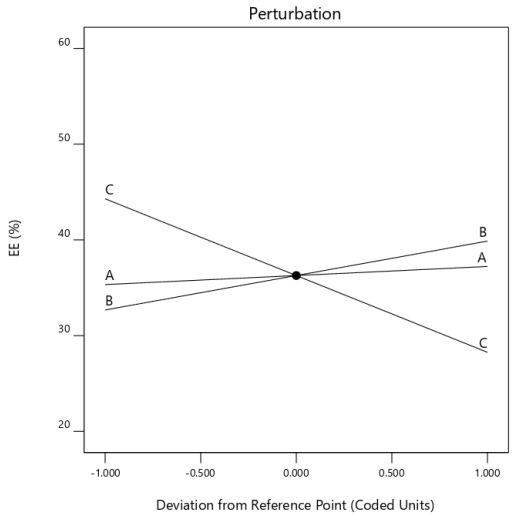


Figure 3. Perturbation plot displaying the effect of three variables on EE% of Van. H loaded MVLs

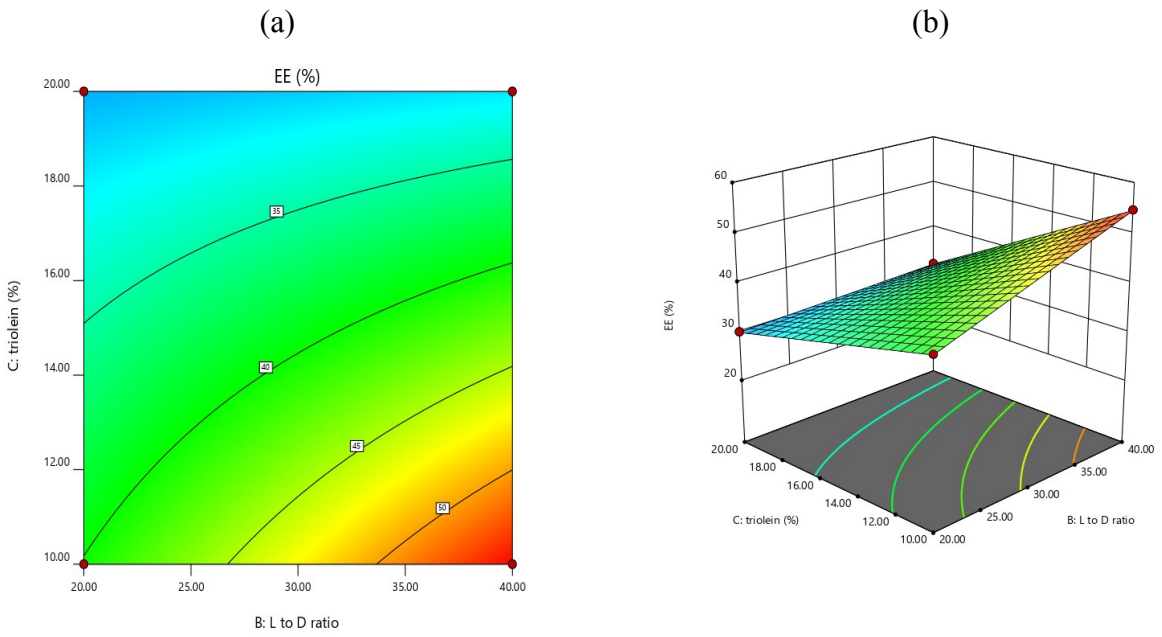


Figure 4. Two-dimensional contour plots(a) and three-dimensional response surface plots (b) showing the combined effects of factor B (L/D) and factor C (To%) on EE%.

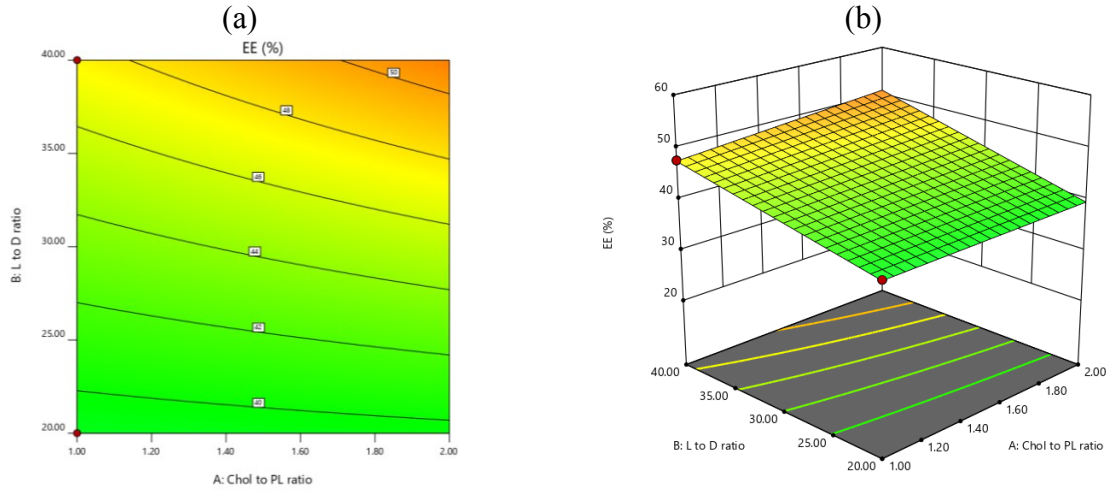


Figure 5. Two-dimensional contour plots(a) and three-dimensional response surface plots (b) showing the combined effects of factor A (Chol/PL) and factor B (L/D) on EE%.

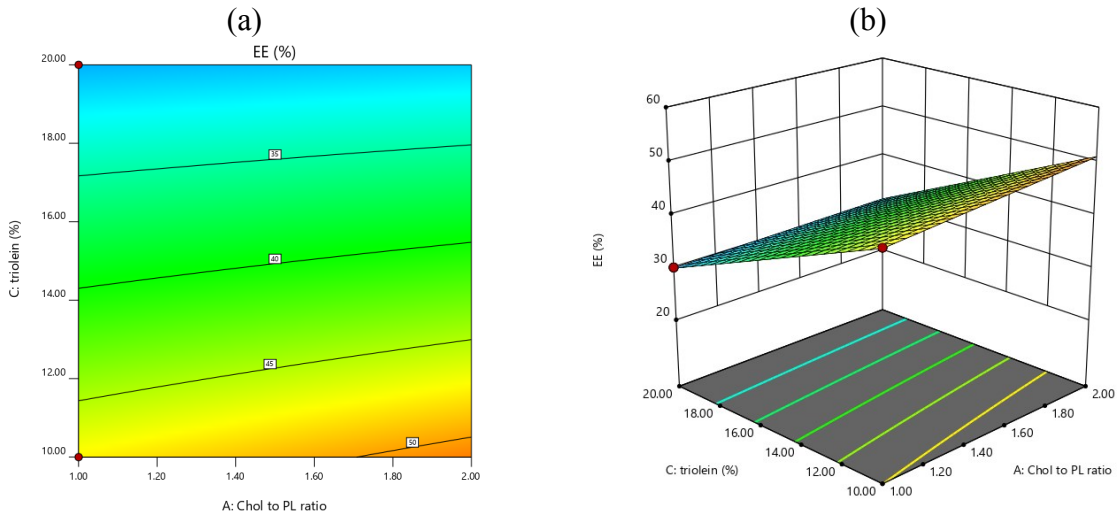


Figure 6. Two-dimensional contour plots (a) and three-dimensional response surface plots (b) showing the combined effects of factor A (Chol/PL) and factor C (To%) on EE%.

3.3. Effects of variables on the drug release rate:

MVL is differentiated from conventional liposomes (unilamellar and multilamellar liposomes) by its unique characteristic structure and composition [25]. MVLs are made up of several nonconcentric aqueous chambers and contain more than 90% of water, so they are a unique carrier system for hydrophilic drugs. One of the specific structural features of MVLs is the presence of a neutral lipid such as triolein, tricaprylene, trilaureine, tributyrine, etc in their structure which plays a significant role in stabilizing the membrane [25,26]. These features allow MVL to overcome the limitations of conventional liposomes such as low entrapment efficiency for water-soluble drugs and drug release after a single breach in the outer membrane. Very low aqueous entrapment is an important barrier to the use of conventional liposomes for hydrophilic drugs. Unlike conventional liposomes, a breach in the outer membranes of this system leads to the release of the encapsulated drug into the external medium, and the release of the drug from the internal vesicles leads to the redistribution of the drug inside the particle instead of releasing from the particle.

As shown in Table 2, the percentage of drug released from the prepared formulations ranged between 53.35% to 84.51% over 24 hours and 68.41% to 94.64% for 48 hours. Polynomial models between the dependent variables (DR24h% and DR48h%) and independent variables were fitted by applying multiple linear regression. For both DR24h% and DR48h % the predicted R^2 is in a reasonable agreement with the adjusted R^2 (0.8748 Vs. 0.9492 and 0.8704 Vs. 0.9388 and respectively). As 'adequate precision' ratio is greater than 4 for both responses, this model can be used to navigate the design space. Through the statistical

analysis for optimization of MVLs, the quadratic model was found significant ($p < 0.05$) for DR24h%(Y2) and DR48h%(Y3) of MVLs. The polynomial equations were created with significant factors which is expressed as follows:

2. Y2 (DR24h%) = + 69.35 + 1.62 A – 7.09 B – 3.50 C – 0.1781 AB + 2.09 AC –0.9087 BC

3. Y3 (DR48h%) = + 82.94 + 1.37 A – 6.63 B – 4.05 C + 1.54 AC –0.6575 BC

As indicated by the equation and the Pareto plots (Fig 2 b and c), the variable B (L/D ratio) as the most effective factor, exhibits a negative effect on the release rate which means increasing the B level leads to a decrease in the release rate. This observation is in agreement with the study of Johenston et al., in which the half-times for doxorubicin release from large unilamellar vesicles (LUV) was increased more than 6-fold by increasing the D/L ratio from 0.05 (wt/wt) to 0.39 (wt/wt) [27]. The likely explanation is that, with an increase of lipid content, the number of MVL vesicles also increases, resulting in less drug being loaded in each vesicle and consequently the release rate decreases as each vesicle release its content independently of the others [20].
According to equations 2 and 3, factors C (To%) and A (Chol/PL) have a negative and a positive effect, respectively on both 24h and 48h release rate. All these findings are confirmed by perturbation plots which are shown in Figure 7.
To further study the combined effects of independent variables on the release rate, contour plots (2D and 3D) were examined. As revealed in Figure 8 (a and b) and Figure 9 (a and b), by keeping the To% constant at its highest level, drug release rate over 24 and 48 h was slower for greater L/D ratios and lower Chol/PL ratios.

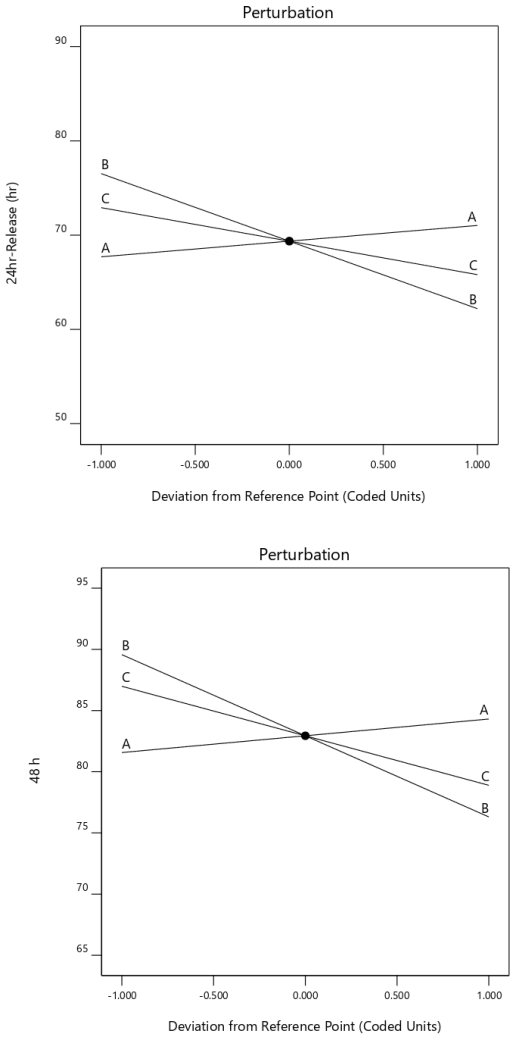


Figure 7. Perturbation plot displaying the effect of three independent variables on the drug release rates after 24 hours (a) and 48 hours (b) from Van. H loaded MVLs

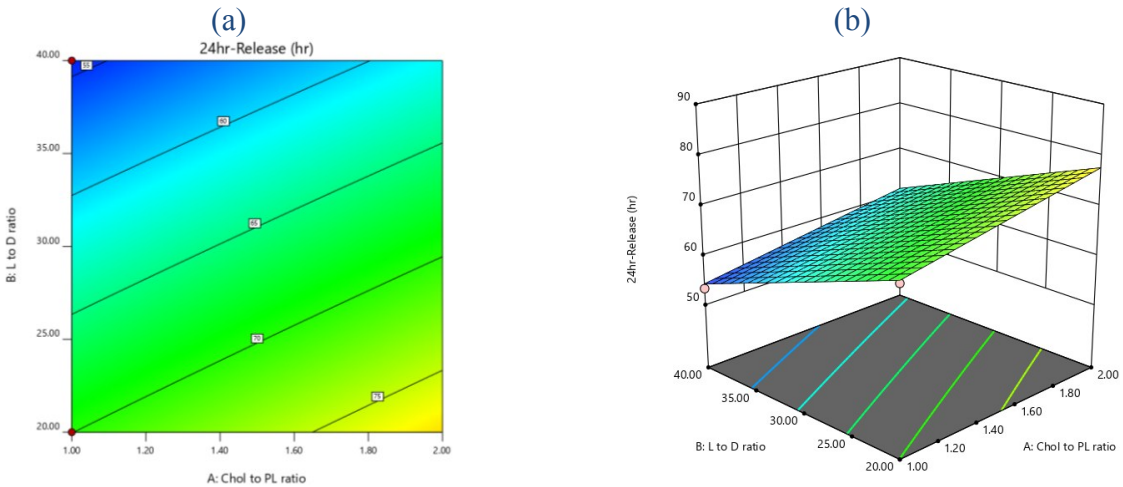


Figure 8. Contour plots showing the combined effects of factor A (Chol/PL) and factor B (L/D) on DR24h%. (a): two dimensional and (b): three dimensional plots

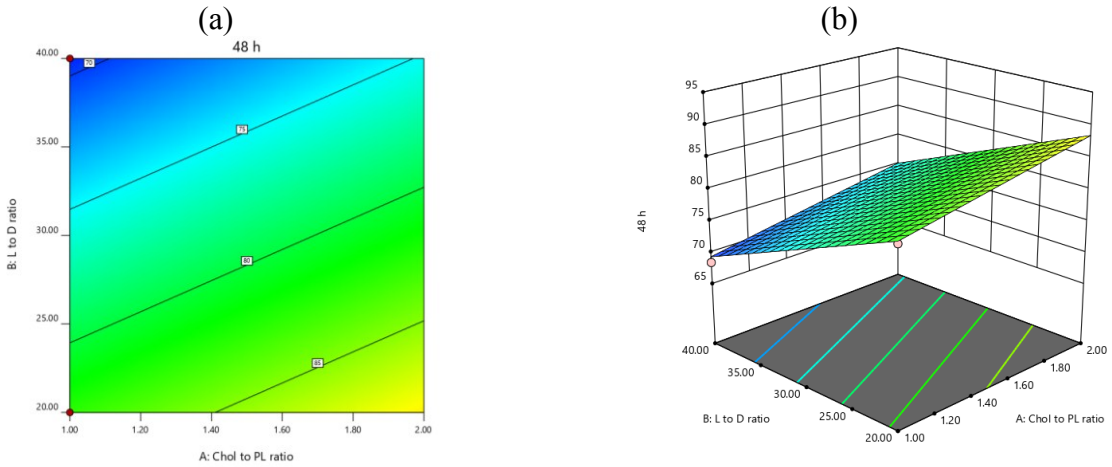


Figure 9. Contour plots showing the combined effects of factor A (Chol/PL) and factor B (L/D) on DR48h%. (a): two dimensional and (b): three dimensional plots

According to previous studies, trioline, a neutral lipid with a long chain of 18 carbons, plays an important role in the stability of the MVL's lipid membrane [28,29]. it is worth noting that the amount of triolein, like other structural components of the MVL, should be within an optimal range, otherwise the surplus of triolein can enter into the water phase in the form of small droplets, and change the acidity of the internal water phase. Accordingly, this process can accelerate the release rate by affecting the osmotic pressure in the internal aqueous phase [18].

As previously discussed, cholesterol plays an imperative role in liposome composition [18]. Due to the location of cholesterol across the phospholipid bilayer of the membrane, its impact on the release rate of hydrophobic and hydrophilic drugs in liposomes has always been discussed extensively. Based on a study by Briuglia et al., Increasing the percentage of Chol accelerates the release rate of hydrophilic drugs [30]. On the contrary, due to the location of the hydrophobic drugs which interact with the long chain of lipids, high percentage of cholesterol will cause a less drug entrapment and a lower release action since the steric hindrance. In fact, given the key role of cholesterol in regulating membrane fluidity, it seems that an extra amount of cholesterol may increase the rigidity of the membrane and cause the lipid membrane to be more fragile [18,30].

3.4. Selection of optimized formulation

In order to estimate the optimization capability of the mathematical models generated by 2^3 factorial design, a numerical optimization model was used. The optimized formula was made by adjusting the determined range for each factor and also minimizing and maximizing each response. The desired range for each factor, including $1< A (Chol/PL) < 3$, $20 < L/D < 40$ and $10 < C (To\%) < 30$ was selected based on the values determined in the experiment design, the results, as well as the possibility of formula stability. Besides, to assess the proper range of responses, EE% and releases were set to the maximum and minimum levels respectively. Finally, the formula suggested by the software was evaluated for morphology, EE %, release rate, and stability.

Various ratios of the independent variables for the optimum formulation were suggested by the software. The solution for the numerical optimization is shown in Figure 10.

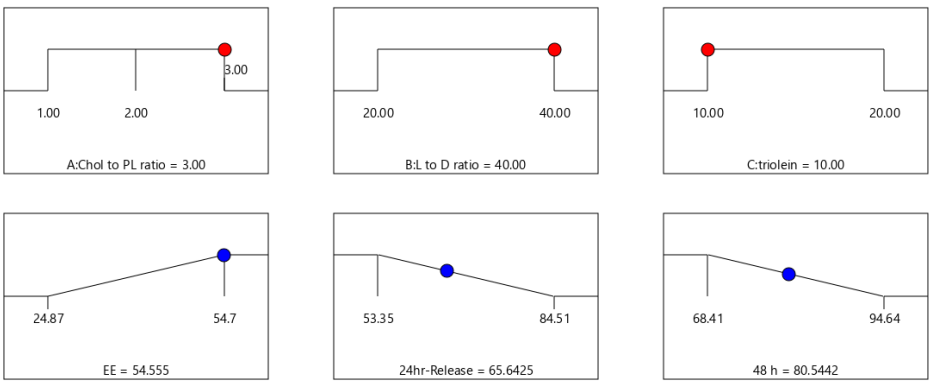


Figure 10. Optimized solution designed by factorial design

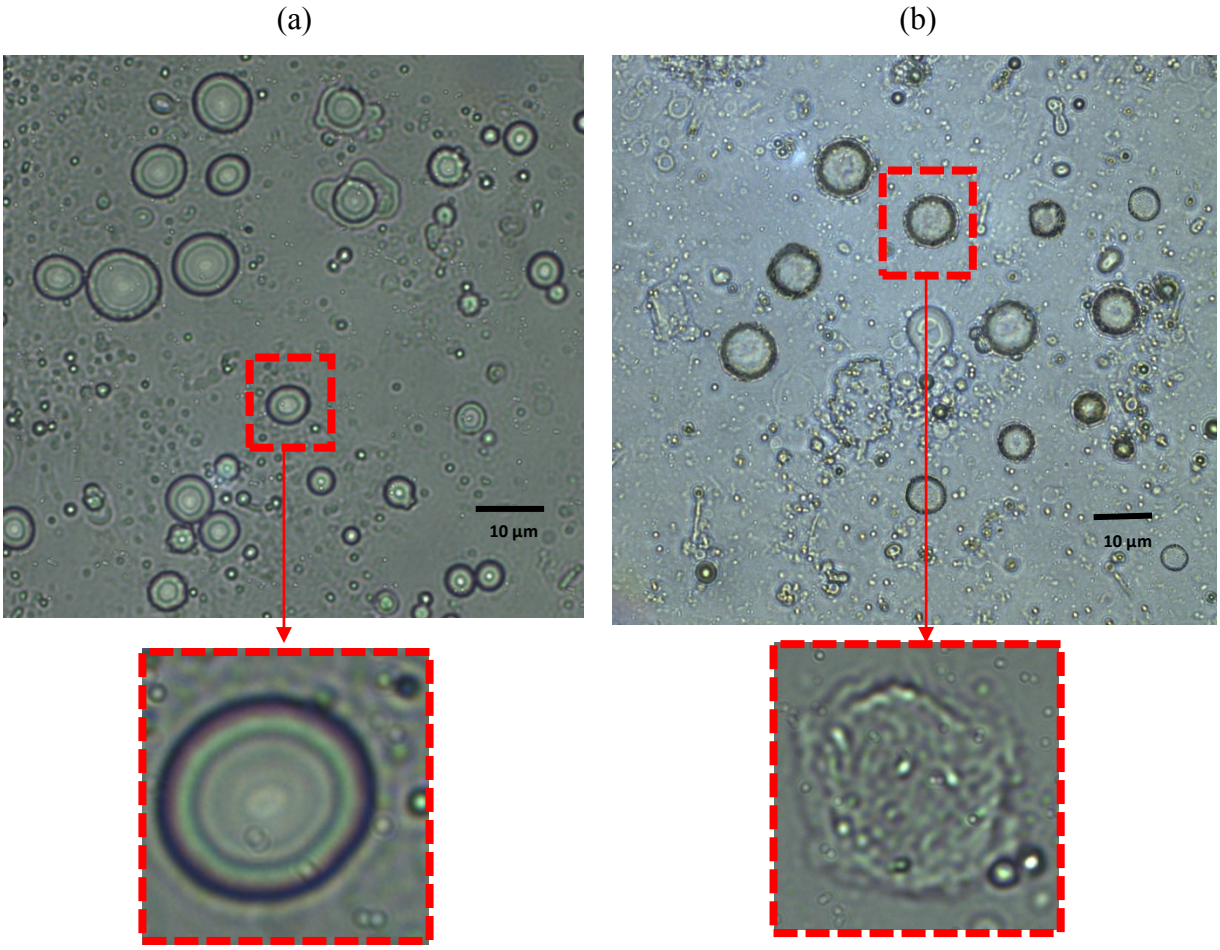


Figure 11. Photomicrograph of (a)multilamellar vesicles (MLVs) and (b) optimized Van H-MVLs through optical microscopy (×400)

As can be seen from this figure, the values selected as the optimal formula correspond to formula #9. Given that the optimal values include the maximum and minimum levels specified in the range of each factor, it was reasonable to check the effect of higher and lower values on the responses as well. Since an L / D ratio above 40 and Chol/PL above 3 are uneconomical and impractical, MVLs with triolein values of 5% and 7% were prepared and evaluated. Due to the important role of triolein in the structural stability of MVLs, especially at the junctions between vesicles, a decrease in the molar ratio of triolein less than 10% had a significant effect on the apparent stability of liposomes. Therefore, large multilamellar structures (MLVs) were observed in microscopic examinations rather than MVL structures (Figure 11 a). Consequently, formula # 9 with a lipid composition of EPC: CHOL: To: DCP (20.75:62.25:10:7 mol ratio) was selected as the optimal formula. This formulation showed an appropriate controlled release profile and the highest encapsulation efficiency (Table 2). Further characterization was performed by evaluating the morphology, size and storage stability of the optimized Van. H-MVLs.

3.4.1. Release profile of the optimized formulation:

As shown in Figure 12, what was observed in the optimal formulation release profile includes an initial burst release followed by a short lag phase and then a secondary sustained release phase. According to a study by Manna et al., this three-phasic release profile is consistent with the features of MVL as a complex dosage carrier. The initial burst is probably due to the release of the free or rapidly dissolved drug from the MVL surface vesicles. Chemical (e.g., surfactants) or physical (e.g., mechanical shear) stressors can accelerate burst release through increasing surface erosion [31]. It is well known that the treatment of osteomyelitis as an infectious disease with the potential for biofilm formation requires fast initial release of antibiotics followed by a sustained one in order to entirely suppress the infection [32-34]. The lag phase is determined to have no significant release of the drug after an initial burst release and before the secondary release phase. This phase occurs due to the temporary discharge of the surface drug and the pursuant slow diffusion through the lipid barriers. It was believed that rearrangement of the

"honeycomb" vesicles played an important role in the "lag" phase. In our study, considering that EPC with a less rigid structure compared to the other phospholipids was used, vesicles rearrangement appears to occur rapidly and therefore the lag phase is short and imperceptible. Moreover, the secondary release phase was associated with continued MVL erosion through lipid hydrolysis and it should be noticed that the continuity and sustainability of the release depends on this stage [31].

In a study by Liu et al. on liposome-loaded vancomycin, nearly 80% of the drug was released within 12 hours [11]. In our study, in addition to a significant reduction in release rate compared to the same study in liposomes, it was also much slower than the free drug (Figure 12).

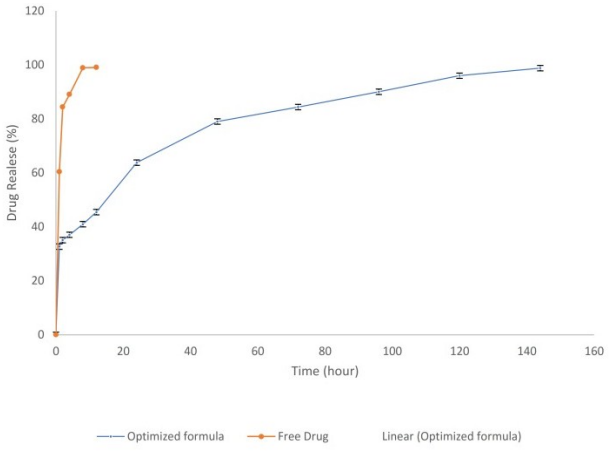


Figure 12. In vitro release profile of free drug and Van H-MVLs in PBS (pH 7.4) (mean ±SD, n=3).

3.4.2. Morphology and size of optimized formula

As shown in Figure 11 b, using optical microscopy (x400), the spherical and multivesicular nature of MVLs was visible. This non-centric granular structure gives the MVLs a "honeycomb" appearance that distinguishes them from other liposomal structures. The particle size of optimized MVLs has also been identified by Mastersizer 2000. The profile showed a single peak with a mean diameter of 9.019 ± 0.26 µm, and the span of 0.188. (Figure 13).

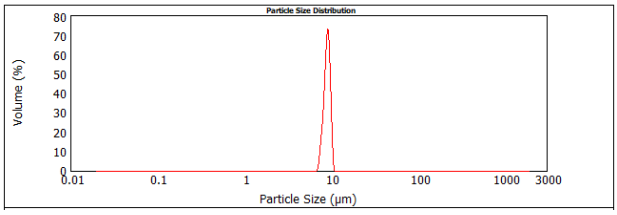


Figure 13. The particle size distribution of Van H-MVLs.

3.4.3. Stability of the optimized formulation

The physicochemical stability of the optimized formulation was studied by monitoring its EE%, size and morphological change for 30 days at 4°C. As indicated in Table 3, proper stability was found with insignificant change in size, morphology and EE%.

Table 3. Stability of the optimized formulation after storage at 4°C for 1 month

Time (week)	Size (µm)	EE%
0	9.01 ± 0.26	54.7 ± 0.6
1	9.12 ± 0.17	53.62 ± 0.3
2	9.08 ± 0.34	53.21 ± 0.4
3	9.14 ± 0.12	54.00 ± 0.7
4	9.20 ± 0.61	52.89 ± 0.5

4. Conclusion

In this study, vancomycin hydrochloride was successfully loaded in multivesicular liposomes by double emulsion method using two-level full factorial design. All prepared MVLs were thoroughly characterized. In microscopic examinations, MVLs with expected and distinguishable appearance from other common liposomes were observed. For the optimized formulation, the average drug encapsulation efficiency and the mean particle diameter were 54.7 ± 0.3% and 9.019 ± 0.26 µm, respectively and the distribution was relatively homogenous. A sustained and slower drug release rate was shown compared to the free drug as well as previously reported Van. H loaded classic liposomes [11]. These findings suggest that Van H-MVLs might have the potential to be used in the local treatment of chronic osteomyelitis as a biocompatible drug carrier with a high antibiotic entrapment capacity as well as controlled drug release.

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Conflict of interest

The authors report no conflicts of interest.

Ethics

Ethics Code: IR.SBMU.RETECH.REC.1399.1261

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