ORIGINAL ARTICLE

QUERCETIN-LOADED LIPOSOMES: FORMULATION OPTIMIZATION THROUGH A D-OPTIMAL EXPERIMENTAL DESIGN

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Abstract

This study aimed to investigate the influence of formulation factors on the physico-chemical properties of quercetin-loaded liposomes and optimize the preparation conditions. An experimental design with three factors and three levels was used. The variables were dipalmitoylphosphatidylcholine (DPPC) concentration, DPPC:Cholesterol (CHOL) molar ratio and quercetin (QU) concentration. The responses were the mean diameter, polydispersity index (PdI) and drug encapsulation efficiency. All samples were prepared according to the experimental design matrix consisting of 17 experiments. Results showed that DPPC concentration and DPPC:CHOL molar ratio had a strong influence on liposome size and PdI. Higher DPPC concentrations led to larger particles and PdI values, but a further increase of DPPC concentration produced a reduction in size and lower PdI. Increasing the lipid ratio caused a decrease in size, but a somewhat higher PdI. QU concentration only slightly influenced particle size and PdI. The degree of QU loading depended on the factors assessed. Increasing DPPC concentration and lipid ratio significantly enhanced QU entrapment. However, higher QU concentrations had a negative effect on drug entrapment. Based on this, an optimized formulation was determined, prepared and analysed. The overall results showed that DPPC concentration and lipid ratio were the main factors influencing particle size, while entrapment efficiency was affected predominantly by QU concentration.

Rezumat

Acest studiu a urmărit influența factorilor de formulare asupra unei metode de preparare a lipozomilor și optimizarea condițiilor de preparare a lipozomilor cu cvercetină. A fost utilizat un plan experimental cu trei factori și trei niveluri. Factorii au fost concentrația de dipalmitoilfosfatidilcolină (DPPC), raportul molar DPPC:Colesterol (CHOL) și concentrația de cvercetină (QU). Răspunsurile au fost diametrul mediu, indicele de polidispersie (PdI) și eficiența încorporării. Probele au fost preparate conform matricei planului experimental ce a cuprins 17 experimente. Conform rezultatelor, concentrația DPPC și raportul molar DPPC:CHOL au influențat puternic diametrul și PdI. Creșterea concentrației DPPC a condus la dimensiuni și PdI mai mari, însă creșterea suplimentară a concentrației DPPC a determinat reducerea acestora. Prin creșterea raportului lipidelor s-a produs reducerea dimensiunii și creșterea ușoară a PdI. Concentrația QU nu a influențat semnificativ diametrul și PdI. Eficiența încorporării a fost influențată de factorii analizați. Prin creșterea concentrației DPPC și raportului lipidelor a fost îmbunătățită eficiența încorporării. Totuși, creșterea concentrației QU a avut un efect negativ asupra încapsulării substanței. Pe baza acestora, a fost stabilită, preparată și analizată o formulare optimizată. Per ansamblu, rezultatele obținute au indicat că principalii factori ce au influențat dimensiunea particulelor au fost concentrația DPPC și raportul lipidelor, în timp ce eficiența încorporării a fost afectată în special de concentrația QU.

Keywords: quercetin, liposomes, experimental design, optimization

Introduction

Liposomes are spherical vesicles consisting of one or several lipid bilayers which delineate an aqueous cavity [10]. The main structural components of liposomes are natural phospholipids or lipids such as egg or soybean phosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), similar to those found in biological membranes [16, 24]. Liposome formulations also comprise cholesterol as the most commonly used sterol. Due to its shape and solubility, cholesterol fills in the empty spaces between the phospholipid molecules, anchoring them more strongly into the liposomal membrane and thus leading to a more stable structure [23].

In the last decades, liposomes have received considerable attention due to their particular characteristics such as high biocompatibility, low toxicity, ability to encapsulate both hydrophilic and

hydrophobic compounds [3, 13, 16]. As drug delivery systems, liposomes have been proposed for transporting different therapeutical agents for cancer treatment, vaccine immunization, gene therapy, radiopharmaceuticals for diagnostic imaging and cosmetic formulations [7, 25].

Quercetin (3,3',4',5,7-pentahydroxyflavone, QU) is a natural flavonoid ubiquitously present in the plant kingdom. It is considered to be one of the best antioxidant flavonoids due to the high number and position of hydroxyl groups, and conjugated π orbitals [5]. It has been shown that QU can prevent or protect against damage caused by free radicals in various ways [17]. One way is by directly scavenging free radicals, leading to more stable and less reactive radicals. Other mechanisms include modulation of gene expression, interactions with different biological enzyme systems and inhibition of lipid peroxidation [1, 15]. Also, QU is particularly known for its ability to chelate transition metal ions such as iron ions [12]. Researches have revealed that QU exhibits different biological effects, including anti-inflammatory, antiallergic, antimicrobial [15], immune-modulatory, antiproliferative and antitumoral effects [22].

In spite of its numerous biological activities and potential applications in the prevention or treatment of human diseases, the use of QU is limited by its low water solubility, high rate of metabolism, rapid clearance from plasma and cells and low bioavailability [24]. These problems can be overcome by developing a liposomal delivery system to solubilize QU, improve its pharmacokinetic and pharmacological properties [8, 14] as liposomes supposedly facilitate intracellular delivery and prolong the retention time of encapsulated drugs inside cells [24].

One goal in the development of drug delivery systems, including liposomes, is to incorporate a sufficient amount of drug in order to assure an optimum concentration at the site of action, and thus therapeutic effectiveness. To achieve optimum encapsulation of a drug into liposomes, parameters influencing both the carrier and the drug such as liposome size, surface charge, lipid composition, bilayer rigidity, need to be considered during the early stages of development [19].

Experimental design methodology is a strategy that allows to study different variables simultaneously, the relationship between them and their influence on different experimental responses, through running a small number of experiments [6]. This technique can be successfully used to optimize liposome preparation processes and conditions [11].

The main objective of this work was to study the influence of three formulation factors on the characteristics of QU-loaded liposomes through experimental design. It involved formulation designs and analysis of response surface plots in order to establish the effects of experimental factors and to obtain an appropriate formulation with target goals.

Materials and Methods

Materials

QU and cholesterol (CHOL) were purchased from Sigma-Aldrich (Germany). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Lipoid GmbH (Germany). All other chemicals used were of analytical grade and the solvents were of HPLC grade.

Methods

Experimental Design. In order to study the influence of different formulation factors on the preparation process of QU-loaded liposomes, a D-Optimal Experimental Design with three factors and three levels has been developed using Modde 10 Software (Umetrics, Sweden) [9]. The selected independent variables (formulation factors) were as follows: DPPC concentration (X_1), DPPC:CHOL molar ratio (X_2) and QU concentration (X_3). The three independent variables as well as their variation levels are presented in Table I.

Table I

Independent variables and their levels of variation

Independent variable	Symbol	Level of variation		
		-1	0	+1
DPPC concentration (mM)	X_1	10	50	90
DPPC:CHOL molar ratio	X_2	1	5.5	10
QU concentration (mM)	X_3	2	6	10

The levels of each factor were set based on preliminary experiments and the feasibility of preparing QU-loaded liposomes at these values. The dependent variables (responses) were expressed in terms of size (Y_1), polydispersity index (PdI, Y_2) and encapsulation efficiency (Y_3). The constructed design matrix comprised 17 experiments. Details of the design matrix are shown in Table II.

Table II

Design Matrix

Exp. No.	Exp. Name	Run Order	X_1	X_2	X_3		Exp. No.	Exp. Name	Run Order	X_1	X_2	X_3
1	N1	14	10	1	2		10	N10	2	90	5.5	6
2	N2	17	90	1	2		11	N11	9	50	1	6
3	N3	7	10	10	2		12	N12	4	50	10	6
4	N4	8	90	10	2		13	N13	6	50	5.5	2
5	N5	5	10	1	10		14	N14	10	50	5.5	10
6	N6	16	90	1	10		15	N15	15	50	5.5	6
7	N7	11	10	10	10		16	N16	1	50	5.5	6
8	N8	13	90	10	10		17	N17	3	50	5.5	6
9	N9	12	10	5.5	6							

X_1 – DPPC concentration; X_2 – DPPC:CHOL molar ratio; X_3 – QU concentration

Preparation of QU-loaded liposomes. QU-loaded liposomes were prepared by the film hydration method first described by Bangham et al [2] with slight modifications. Briefly, the corresponding amounts of DPPC, CHOL and QU were dissolved in 5 mL of methanol. The solution was then transferred to a 250 mL round bottom flask. Subsequently, the organic solvent was removed under reduced pressure at 40°C by a rotary evaporator (Heidolph, Germany), rotating at 100 rpm, yielding a thin lipid film on the sides of the flask. The lipid film was thoroughly dried to remove any residual traces of the organic solvent by maintaining the flask under a nitrogen gas flow for one hour. Afterwards, the lipid film was hydrated by adding 2 mL phosphate buffered saline (PBS, pH=7.4) and the mixture was kept under continuous stirring at 40°C for 30 minutes.

Purification and size reduction of QU-loaded liposomes

Immediately after preparation, the QU-loaded liposomes were centrifuged at a speed of 10000 rpm for 15 minutes (Sigma, Germany). The supernatant containing the nonentrapped drug was separated from the lower remaining sediment represented by the QU-loaded liposomes [20]. A volume of fresh PBS was added to yield 2 mL of liposome dispersion, and then the mixture was vortexed until homogenization. In order to reduce their size, the liposomes were extruded three times through 0.8 μm and 0.2 μm, respectively, pore size polycarbonate membranes (Whatman International Ltd, UK) using a LiposoFast LF-50 extruder (Avestin Europe GMbH, Germany).

Determination of particle size and PdI. Particle size and polydispersity of QU-loaded liposomes were determined by dynamic light scattering using a Zetasizer (Malvern, UK). 50 μL of liposome suspension were dispersed in double distilled water and then analysed. Each sample was measured three times.

Determination of encapsulation efficiency. The liposome dispersion was dissolved in methanol, and the encapsulated QU was determined by HPLC analysis. The encapsulation efficiency (%EE) was calculated from the ratio of the amount of entrapped QU (C_{lipo}) to that initially added (C_{tot}), according to the equation: $\%EE = C_{lipo}/C_{tot}*100$.

Results and Discussion

Preparation and characterization af QU-loaded liposomes. The experimental results concerning particle size, PdI and encapsulation efficiency from the 17 experiments are shown in Table III.

Table III

Observed responses for particle size (Y_1), PdI (Y_2) and encapsulation efficiency (Y_3)

Exp. Name	Y_1	Y_2	Y_3
N1	264.9±0.2309	0.115±0.007	3.21
N2	254.9±0.3512	0.085±0.036	24.09
N3	225.5±3.156	0.152±0.018	4.10
N4	243.7±6.108	0.170±0.019	53.71
N5	215.1±2.248	0.169±0.009	1.15
N6	247.7±1.701	0.083±0.023	2.00
N7	213.9±1.453	0.161±0.034	1.86
N8	234.2±4.801	0.196±0.089	27.19
N9	218.2±1.484	0.136±0.009	3.46
N10	247.4±1.552	0.127±0.028	72.21
N11	288.3±2.495	0.235±0.006	4.84
N12	224.9±1.474	0.091±0.036	66.50
N13	283.3±4.782	0.258±0.039	71.80
N14	264.1±7.687	0.300±0.017	19.50
N15	239.4±2.211	0.185±0.016	32.30
N16	255.0±3.553	0.254±0.053	36.41
N17	262.9±3.005	0.273±0.038	32.43

Data are shown as mean ± standard deviation

Experimental Design Analysis. Fitting the model. Data were fitted by means of partial least squares

(PLS) and were analysed using the statistical module of the Modde 10 Software. In order to fit the experimental data to the desirable model and to check the validity of the experimental design, R^2 and Q^2 were calculated and analysis of variance (ANOVA) was performed. The fitted model is considered adequate if the model is significant ($p<0.05$) and the lack of fit is not significant ($p>0.05$) [4, 18].

R^2 and Q^2 provided the best information on fitting the model. The model validity indicates if the model is appropriate and if the right type of model was chosen from the beginning. Reproducibility reflects a summary of variability [4, 9]. The overall results of the model fitting are summarized in Figure 1. According to Figure 1, all three responses, particle size, PdI and encapsulation efficiency, are well fitted and predicted by the model. The results show that the relationship between the variables and responses was well described by the chosen model, thus indicating a good and valid model with good predictive power.

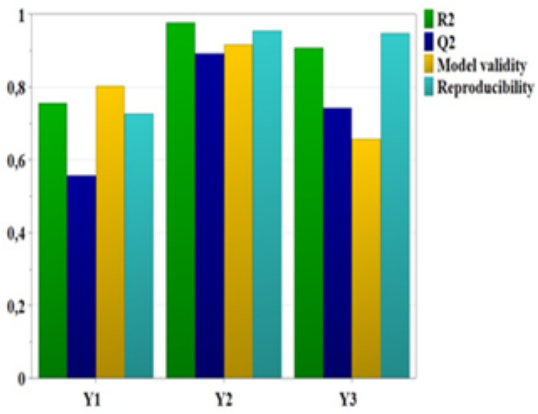

Figure 1.
Summary of fit for the experimental data

The analysis of variance (ANOVA) indicates if the variance of the results is due to variations in the formulation factors or is determined by experimental errors [19]. In all cases, p-values for the model were lower than 0.05, while those for lack of fit were greater than 0.05. The results for the ANOVA test indicate that the data obtained for all responses were good, that is, the model represented the data accurately.

In order to study the effect of the formulation factors on a response, three-dimensional response surface curves were plotted. These plots were used to describe the interaction of two independent variables on the response at one time, while keeping the third variable constant, at its middle level [26].

Influence of formulation factors on particle size (Y_1). The equation generated by PLS regression that shows a correlation and emphasises the influence of formulation factor on the particle size response (Y_1) is the following:

$$Y_1=2.413+0.0136X_1-0.0177X_2-0.0137X_3-0.0251X_1^2+0.0065X_1X_3+0.0049X_2X_3 \quad (1).$$

Particle size is a critical factor for nanoscaled drug delivery systems as it controls the drug's release kinetics. Usually, the smaller the particles size the faster the release rate [21]. The particles size values for the 17 samples show a narrow variation in response, ranging from a minimum of 213.9 nm to a maximum of 288.3 nm. Figure 2 shows the response surface plots indicating the effects of the formulation factors on particle size.

According to the results, DPPC concentration and DPPC:CHOL molar ratio had a significant influence on particle size. As shown in Figure 2A, an increase of DPPC concentration led to an increase in particle size, but a further increase of the phospholipid concentration had an opposite effect causing a reduction in size. It might be due to the fact, a greater amount of DPPC will probably determine the lipid bilayer to reorganize, thus resulting a larger number of liposome particles, but smaller in size.

According to Figure 2B, increasing DPPC:CHOL ratio led to a decrease in particle size. The lipid bilayer's formation and its fluidity depended on the amount of cholesterol used, since it inserts between the phospholipid molecules, changing their mobility in the bilayer, thus reinforcing the membrane's stability [11]. The presence of cholesterol in the lipid bilayer is a critical factor, as it determines the liposome's flexibility and influences the drug release. This aspect should be taken into consideration particularly in the case of hydrophobic compounds like QU which interact with the lipid membrane [22].

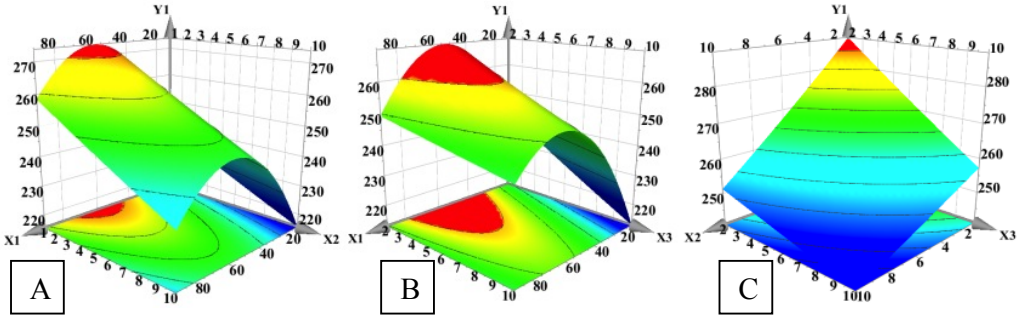

Figure 2.

Three-dimensional response surface plots showing the effect of formulation factors on particle size:
A - DPPC concentration and DPPC:CHOL molar ratio effect; B - DPPC and QU concentration effect;
C - DPPC:CHOL molar ratio and QU concentration effect.

X_1 - DPPC concentration; X_2 - DPPC:CHOL molar ratio; X_3 - QU concentration; Y_1 - particle size; Y_2 - PdI; Y_3 - encapsulation efficiency

The effect of QU concentration on particle size is given in Figure 2C. A slight decrease in liposome size was observed when increasing QU concentration. Due to its lipofilicity, QU molecules are inserted in the lipid bilayer and possibly replace the phospholipid molecules within the liposomes. Thus, the membrane tends to reorganize forming smaller particles.

Influence of formulation factors on PdI (Y_2). The equation generated by PLS regression for PdI response (Y_2) is as follows:

$$Y_2=0.270-0.0056X_1+0.0223X_2+0.0101X_3-0.0821X_1^2+0.0132X_1X_2 \quad (2).$$

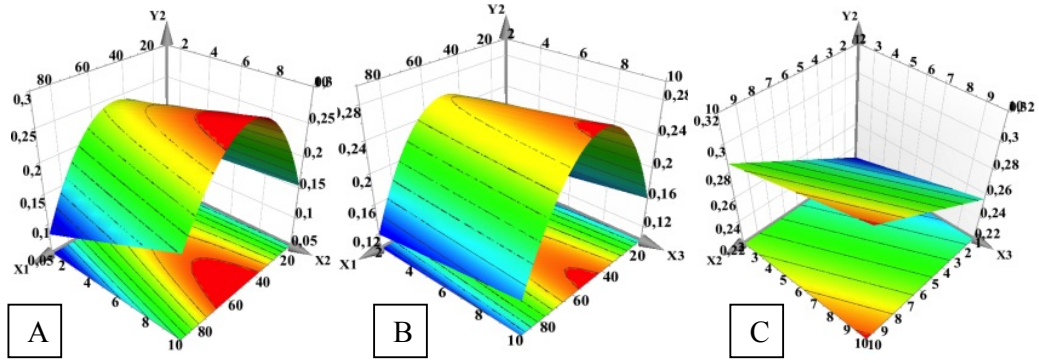

Figure 3.

Three-dimensional response surface plots showing the effect of formulation factors on PdI:
A - DPPC concentration and DPPC:CHOL molar ratio effect; B - DPPC and QU concentration effect;
C - DPPC:CHOL molar ratio and QU concentration effect.

X_1 - DPPC concentration; X_2 - DPPC:CHOL molar ratio; X_3 - QU concentration; Y_1 - particle size; Y_2 -PdI; Y_3 - encapsulation efficiency

PdI is an important parameter which is used to describe variation of particle size in a population of particles. When the PdI value is close to 1, the size range is wide. Generally, a value closer to 0 is desired [21].

In all the tested samples, the PdI was lower than 0.5, and varied between 0.083 and 0.300. The response surface plots for the PdI are shown in Figure 3. Increasing DPPC concentration led to an increase of the PdI up to a point beyond which index values decreased. Furthermore, by increasing DPPC:CHOL molar ratio and drug concentration, the PdI increased as well.

Influence of formulation factors on encapsulation efficiency (Y_3). For various formulation factors' combinations, the encapsulation efficiency of QU into liposomes varied widely from 1.15% to 72.21%. In this case, the equation generated by PLS regression is:

$$Y_3=1.574+0.3987X_1+0.2562X_2-0.1966X_3-0.2241X_1^2-0.2028X_2^2-0.0796X_3^2 \quad (3).$$

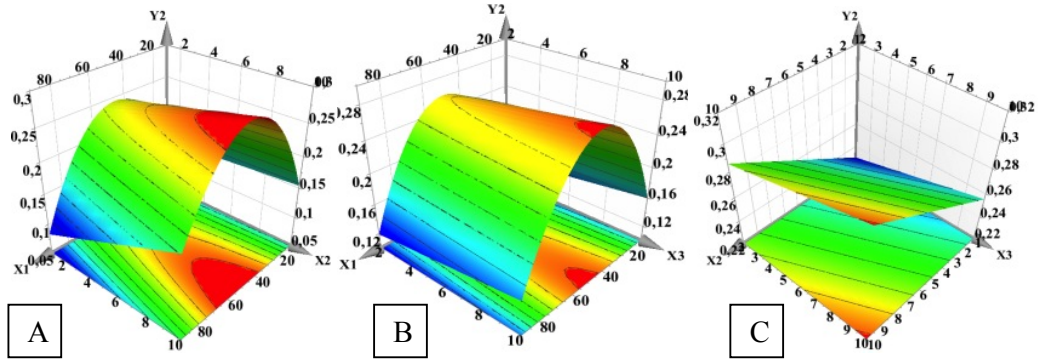

Figure 4.

Three-dimensional response surface plots showing the effect of formulation factors on encapsulation efficiency: A - DPPC concentration and DPPC:CHOL molar ratio effect; B - DPPC and QU concentration effect; C - DPPC:CHOL molar ratio and QU concentration effect.

X_1 - DPPC concentration; X_2 - DPPC:CHOL molar ratio; X_3 - QU concentration; Y_1 - particle size; Y_2 -PdI; Y_3 - encapsulation efficiency

Figure 4A and B reveal that encapsulation efficiency first increased when increasing DPPC concentration and DPPC:CHOL molar ratio, and then decreased. This can be attributed to several factors. Firstly, the increase might be due to the fact that a greater amount of DPPC provides additional space for drug molecules to entrap. Secondly, the addition of cholesterol gives the bilayer a certain rigidity. A small DPPC:CHOL molar ratio causes decrease in entrapment probably because cholesterol might be replacing QU in the bilayer [22]. Likewise, a small amount of cholesterol resulted in lower encapsulation efficiency most likely because due to its increased fluidity, the lipid bilayer could only accommodate a certain amount of QU. Encapsulation efficiency is expected to increase with the increase in QU concentration. However, contrary to this, by increasing the drug concentration, a decrease in entrapment was registered (Figure 4C). As the entrapment efficiency is calculated as a ratio between the amount of drug entrapped and the amount of drug initially added, it is only obvious that the encapsulation efficiency would be lower at higher QU concentrations. On the one hand, a high encapsulation efficiency can be due to the hydrophobic nature of the lipids that make up the liposomes, which therefore easily entrap hydrophobic compounds like QU. On the other hand, because of its hydrophobicity, the loss of QU to the external aqueous phase is minimal.

Optimization. The optimization process was carried out in order to optimize the three responses simultaneously. Therefore, particle size (Y_1) and PdI (Y_2) were minimized, while encapsulation efficiency was maximized (Y_3). In order to evaluate the predictive power of this model, QU-loaded liposomes were prepared under the optimum conditions which were as follows: DPPC concentration of 89.99 mM, DPPC:CHOL molar ratio of 5.31 and QU concentration of 3.32 mM. The predicted and actual experimental values of the three responses are shown comparatively in Table IV.

Table IV

Predicted and experimental values of the QU-loaded liposomes' characteristics, under predicted optimum conditions

Response	Constraint	Predicted value	Experimental value	Bias (%)
Y_1 Particle size (nm)	Minimize	205.21	211.90±0.50	+3.26
Y_2PdI	Minimize	0.06	0.05±0.01	-8.47
Y_3 Encapsulation efficiency (%)	Maximize	70.00	61.25	-12.50

The experimental values were close to the predicted ones, with low percentage bias (under ±15% set point), thus suggesting that the optimized formulation was reliable and affirming the validity of the proposed model.

Conclusions

This study evaluated the influence of three formulation factors on QU-loaded liposomes' characteristics by using a D-Optimal Experimental Design. It was concluded that DPPC concentration, DPPC:CHOL molar ratio and QU concentration played a significant role in controlling the particle size, PdI and encapsulation efficiency of QU-loaded liposomes.

Starting from the results, the optimum preparation conditions for QU-loaded liposomes were set, and consecutively the optimum formulation was

prepared. A good correlation between the model predicted and actual experimental response values was obtained for the optimum formulation. Therefore, the optimized preparation conditions and proposed model were very reliable.

In conclusion, a D-optimal Experimental Design was successfully used in order to obtain QU-loaded liposomes with optimized characteristics.

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