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Response Surface Methodology for the Optimization of Lactoferrin Nano-Liposomes

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Abstract: The purpose of this study was to optimize the formulation of lactoferrin nano-liposomes using response surface methodology. Response surface methodology based on central composite rotatable design has been successfully used to model and optimize biochemical and biotechnological processes. The mass ratio of phosphatidylcholine and cholesterol (1-8), lactoferrin concentration (2-15 mg/mL), tween80 (0.5-2.5 mg/mL) and the volume ratio of organic phase and aqueous phase (2-10) were selected as independent variables with encapsulation efficiency and particle size as dependent variables. For each response, a 2^{nd}-order polynomial model was developed using multiple linear regression analysis. Applying a desirability function method the optimum parameters were: phosphatidylcholine to cholesterol mass ratio of 5.77, lactoferrin concentration of 11.71 mg/mL, tween80 of 1.18 mg/mL and organic phase to aqueous phase volume ratio of 4.59. At this optimum point, particle size and encapsulation efficiency were found to be 214 nm and 60.21%, respectively. Furthermore, leakage ratio of nano-liposomes was used to determine the influence of storage period.

Keywords: Encapsulation efficiency, lactoferrin, nano-liposomes, particle size, response surface methodology, stability

INTRODUCTION

Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein of the transferring family, which was first isolated from milk by Groves (1960). Lf is abundant in milk and other biological fluids, such as tears, saliva, mucous, pancreatic juice and bile and so on. Lf is a protein with multiple biological functions and it is not only involved in iron transport, but also has immune response, anticarcinogenic activities, antioxidant activities, antimicrobial activities (Baker and Baker, 2005; García-Montoya *et al.*, 2012; González-Chávez *et al.*, 2009; Wakabayashi *et al.*, 2006). However, lactoferrin molecular weight is greater than thousands of Dalton and poor through the biofilm (Wang, 2008). As a vital role in food, proteins are able to form gels and emulsions, which allow them to be an important material for the encapsulation of bioactive compounds (Chen *et al.*, 2006; Wang *et al.*, 2011). Thus, particle delivery systems for nutrients in food are popular (Fathia *et al.*, 2012).

There has been considerable interest in liposome (Rongen *et al.*, 1997), as they may be used for protection in food and pharmacy system (Felnerova *et al.*, 2004; Leserman, 2004; Torchilin, 2005). Liposome are spherical vesicles with a diameter ranging from 20 nm to a few thousands nm, which are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar head groups of the lipids orienting towards the extra vesicular solution and inner cavity (Edwards and Baeumner, 2006; Lorin *et al.*, 2004). The liposome enhance the stability of the encapsulated material by protecting them from the environment (Mozafari *et al.*, 2008) and influence material deposition into the deeper skin layers (Verma *et al.*, 2003).

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques by analyzing the response surface contour to find optimal process parameters and using multiple quadratic regression equation to fit between the factors and the response function. RSM is a useful technology in developing processes and optimizing their performance (Raissi, 2009). Response surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food (Liyana-Pathirana and Shahidi, 2005; Pompeu *et al.*, 2009; Wang *et al.*, 2007).

The main objective of this study was to study the ratio of phosphatidylcholine and cholesterol (w/w), lactoferrin concentration (w/v), tween80 (w/v) and the ratio of organic phase and aqueous phase (v/v) on the Encapsulation Efficiency (EE) and Particle Size (PS) and to find out the optimal conditions for preparing the lactoferrin nano-liposomes using RSM. Furthermore, leakage ratio of nano-liposomes was used to determine the influence of storage period.

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MATERIALS AND METHODS

Materials: Phosphatidylcholine (PC) was purchased from Beijing Shuangxuan Microbe Culture Medium Products Factory (Beijing, China). Cholesterol (CH), pepsin and steapsin were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Lactoferrin was purchased from Seebio Company (Shanghai, China). Chloroform, diethyl ether and Tween 80 were obtained from Hangzhou Jiachen Chemical Company. All chemicals were of reagent grade and used without further purification.

Preparation of lactoferrin nano-liposomes: Lactoferrin nano-liposomes were prepared by reverse-phase evaporation method (Szoka and Papahadjopoulos, 1978). Briefly, a certain amount of PC and CH were dissolved in chloroform-diethyl ether and lactoferrin was dissolved in phosphate buffer solution (pH7.4). The organic phase was mixed with the aqueous phase using probe sonication for 5 min. The mixture was placed in a round-bottom flask and a gel was formed by evaporating the organic solvent under reduced pressure at 35°C using a rotary evaporator. Then 30 mL phosphate buffer solution (0.20 M, pH 7.4, PBS) containing tween 80 was added and evaporated for another 20 min.

Particle size: The particle size was measured by Mastersizer 2000 instrument (Malvern), equipped with Hydro Mu dispersing unit (Malvern). Measurements were taken in the range between 0.1 and 1000 μm,

Table 1**:** Levels of factors used in CCRD

	Independent variable level				
Independent variables	Low	Center	High	Axial (-α)	Axial (+α)
PC: CH (w: w)	2.75	4.5	6.250	1	8
Lactoferrin concentration (w/v)	5.25	8.5	11.75	2	15
Tween 80 (w/v)	1	1.5	2	0.5	2.5
Organic phase: aqueous phase (v:v)	4	6	8	2	10

Table 2**:** Scheme of CCRD with the results of responses on four independent factors

	Independent variable			Response variable		
Run	PC: CH (w:w)	Lactoferrin concentration (w/v)	Tween 80 (w/v)	Organic phase: aqueous phase (v:v)	Particle size (Y_1/nm)	EE (Y_2/%)
1	2.75	11.75	1.00	4.00	233	50.41
2	4.50	8.500	1.50	10.0	298	58.30
3	2.75	11.75	2.00	8.00	281	11.84
4	4.50	2.000	1.50	6.00	301	40.96
5	2.75	5.250	2.00	8.00	264	10.72
6	6.25	11.75	2.00	8.00	239	19.24
7	6.25	11.75	1.00	8.00	238	55.01
8	6.25	5.250	2.00	8.00	275	51.09
9	6.25	11.75	2.00	4.00	225	31.31
10	4.50	8.500	1.50	6.00	213	49.67
11	8.00	8.500	1.50	6.00	230	52.23
12	2.75	5.250	2.00	4.00	226	2.730
13	6.25	11.75	1.00	4.00	224	65.58
14	6.25	5.250	1.00	8.00	287	58.69
15	4.50	8.500	0.50	6.00	280	60.09
16	4.50	8.500	1.50	6.00	220	53.36
17	4.50	15.00	1.50	6.00	237	40.10
18	4.50	8.500	1.50	6.00	218	50.36
19	4.50	8.500	1.50	6.00	224	46.54
20	4.50	8.500	1.50	6.00	225	47.57
21	4.50	8.500	2.50	6.00	257	0.900
22	2.75	11.75	1.00	8.00	278	56.90
23	2.75	11.75	2.00	4.00	224	5.030
24	4.50	8.500	1.50	6.00	224	49.67
25	2.75	5.250	1.00	8.00	267	34.79
26	4.50	8.500	1.50	6.00	220	52.02
27	4.50	8.500	1.50	2.00	223	43.36
28	6.25	5.250	1.00	4.00	290	53.96
29	2.75	5.250	1.00	4.00	230	30.88
30	6.25	5.250	2.00	4.00	290	38.23
31	1.00	8.500	1.50	6.00	215	5.980

under the following conditions: water refractive index 1.33 and general calculation model for irregular particles. The data obtained were averaged by software (Mastersizer 2000, ver.5.20 from Malvern).

Encapsulation Efficiency determination (EE): The encapsulation efficiency was determined by centrifuge-UV method. Take liposome suspension (500 μL) by spinning at 10000 rpm for 30 min using centrifuge, the protein content of the supernatant was measured by Bradford. The same suspension was ruptured using sufficient volume of ethanol and the total amount of lactoferrin was determined spectrophotometrically. Encapsulation efficiency was calculated using Eq. (1):

$$EE\% = \frac{Q_t - Q_f}{Q_t} * 100 \quad (1)$$

where,
Q_f : The amount of free lactoferrin
Qt : The total amount of lactoferrin present in 500 μL of nano-liposomes

Stability analysis: Lf nano-liposomes were stored at 4 °C in a refrigerator. Take 0.50 mL samples at predetermine intervals. The leakage ratios of samples were calculated and pH was measured by pH meter. Leakage ratios were calculated using Eq. (2):

$$L_O = \left(W_{EE} - W_{EE_t}\right) \Big/ W_{EE} \times 100\% \quad (2)$$

where,
W_{EE} = Encapsulation of preparation
W_{EE_t} = Encapsulation of a certain period of time

Experimental design and optimization: RSM as a generic method for optimization was applied to optimize the formulation of Lf liposome. The optimization was designed based on a four-factor central composite rotatable design with a total of 31 experimental runs that involved 7 replicates at the center points (Myers and Montgomery, 1995). Based on the preliminary experiments and our previous studies, 4 formulation parameters which included PC: CH (x_1), lactoferrin concentration (x_2), tween 80 (x_3) and the ratio of organic phase and aqueous phase (x_4), were identified as key factors responsible for EE and particle size. In view of the feasibility of liposome preparation, the ranges of the four factors were determined as follows: PC: CH (1-8, w/w), lactoferrin concentration (2-15, w/v), tween 80 (0.5-2.5, w/v) and organic phase: aqueous phase (2-10, v/v) (Table 1). The experimental runs for CCRD were shown in Table 2. The response could be related to the selected variables by a 2nd-order polynomial model. In this study, a second-order polynomial Eq. (2) was used to generate response surfaces:

$$\hat{Y}_i = b_0 + \sum_i b_i x_{i\backslash} + \sum_i b_{ii} x_i^2 + \sum_{i \neq j} b_{ij} x_i x_j \quad (3)$$

where,
$\hat{Y}_i$: The predicted responses
x_i and x_j : The coded values of independent variables
b_0 : The intercept coefficient
b_i : The linear coefficients
b_{ii} : The squared coefficients
b_{ij} : The interaction coefficients (Zhang *et al.*, 2007)

Statistical significance of the terms in the regression equations was examined. The significant terms in the model were found by Analysis of Variance (ANOVA) for each response. The adequacy of model was checked accounting for R^2 and adjusted-R^2. The desired goals for each variables and response were chosen. All the independents variables were kept within range while the responses were either maximized or minimized.

RESULTS AND DISCUSSION

Fitting the model: Table 2 showed the combined effects of phosphatidylcholine/cholesterol ratio, lactoferrin concentration, tween 80 and organic phase/aqueous phase ratio on PS and EE. The second-order polynomial response surface model Eq. (3) was fitted to each of the response variables (Y_i). For the corresponding fitting of the explanatory models, the variations of particle size and encapsulation efficiency were analyzed. These analyses indicated that adding terms up to quadratic significantly improved the model (Table 1) and could be the most appropriate model for the two response variables.

Regression analysis and the Analysis of Variance (ANOVA) were used for fitting the model and to examine the statistical significance of the terms. The estimated regression coefficients for the response variables, along with the corresponding R^2, adj-R^2, Coefficient of Variation (CV), F-value and p-value of lack of fit, are shown in Table 3.

The lack of fit shows that the models failed to represent the data in the experimental domain at which points were not included in the regression. The lack of fit illustrated in Table 2, was not significant for the two response surface model, meaning that 2 models represented the data accurately.

In addition, adj-R^2 and Coefficient of Variation (CV) were calculated to check the model adequacy. R^2 values for these response variables were higher than 0.80, indicating the regression models were suitable to

Table 3: ANOVA and regression coefficients of the second-order polynomial model for the response variables (actual values)

		PS (nm)			EE (%)		
Source	DF	Coefficient	S.S.	p-value	Coefficient	S.S.	p-value
Model	14	220.57	24512.5	<0.0001	49.880	10902.16	<0.0001
Linear	1						
b1	1	3.9600	376.040	0.00900	10.930	2866.94	<0.0001
b2	1	-13.130	4134.38	<0.0001	0.5200	6.52000	0.50060
b3	1	-2.8800	198.380	0.04630	-14.770	5233.60	<0.0001
b4	1	14.040	4732.04	<0.0001	2.0800	104.290	0.01410
Quadratic							
b11		0.3700	3.86000	0.76700	-5.4800	858.910	<0.0001
b22		11.990	4112.68	<0.0001	-2.6200	196.940	0.00160
b33		11.870	4027.40	<0.0001	-5.1300	753.450	<0.0001
b44		9.8700	2784.33	<0.0001	-0.0490	0.07000	0.94420
Interaction							
b12		-15.310	3751.56	<0.0001	-4.7400	359.960	0.00010
b13		0.1900	0.56000	0.90990	3.0800	151.840	0.00430
b14		-10.440	1743.06	<0.0001	-1.8900	57.1900	0.05810
b23		0.9400	14.0600	0.57330	-5.3100	450.820	<0.0001
b24		4.5600	333.060	0.01290	-2.4300	94.2400	0.01860
b34		0.0630	0.06300	0.96990	0.6900	7.60000	0.46760
Residual	16		680.460			219.680	
Lack of fit	10		572.750	0.08440		186.180	0.07690
Pure error	6		107.710			33.5000	
Total	30		25192.97			11121.84	
R^2		0.9730			0.9802		
Adj-R^2		0.9494			0.9630		
CV		2.6400			9.3600		

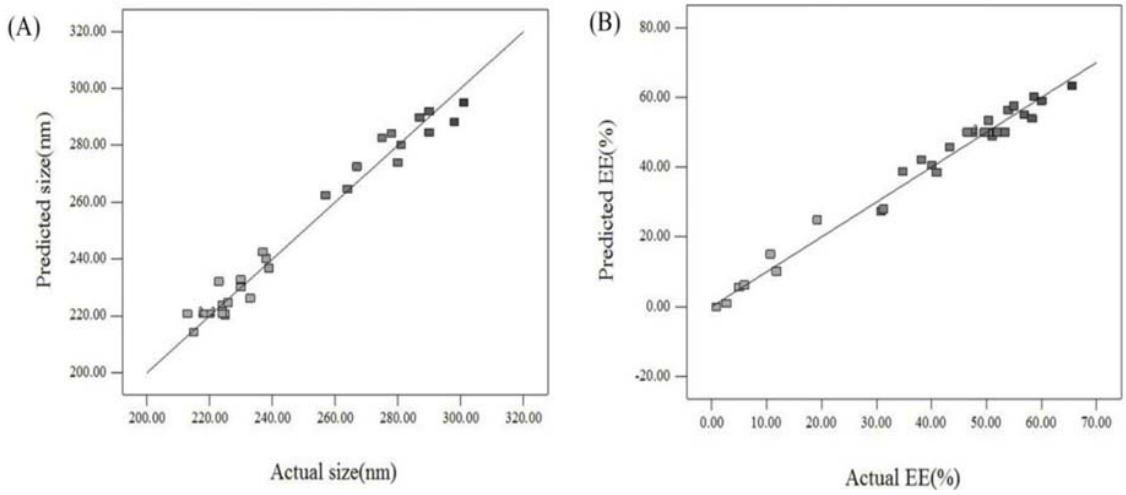
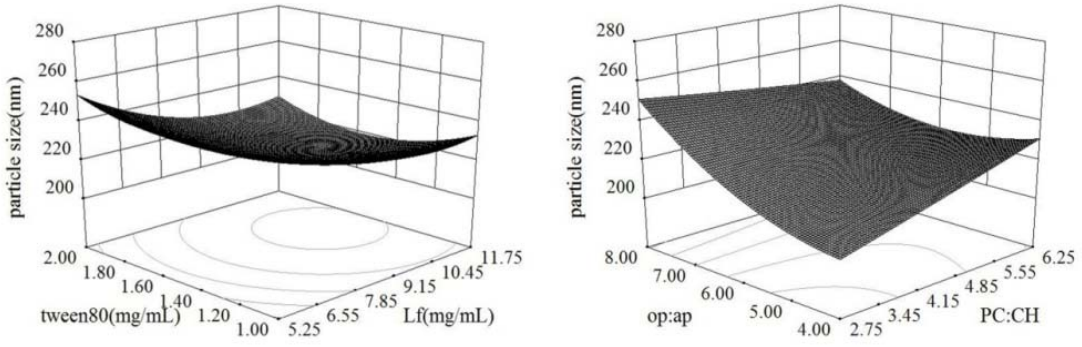


Fig. 1: Comparison between predicted and actual values of particle size and EE of lactoferrin nano-liposomes

explain the behavior, but a large value of R^2 does not always imply the adequacy of the model. Adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significant or not. Thus, it is better to use an adj-R^2 to evaluate the model adequacy. The R^2 values were 0.97 and 0.98 for PS and EE respectively (Table 3). R^2 and adj-R^2 values for the model did not differ greatly; indicating non-significant terms have not been included in the model. As a general rule, a CV higher than 10% indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model. The CV values for PS and EE were found to be 2.64 and 9.36 which represented a better reproducibility and reliability of the conducted experiments.

Figure 1 a, b shows that the polynomial regression model was in good agreement with the experimental results. In this figure, each of the observed values is compared with the predicted value calculated from the



(a) (b)

Fig. 2**:** Response surface for the effect of independent variables on particle size of lactoferrin nano-liposomes: lactoferrin concentration and tween 80 (a, phosphatidylcholine to cholesterol ratio = 4.5 and organic phase to aqueous phase ratio = 6), and phosphatidylcholine to cholesterol ratio and organic phase to aqueous phase ratio (b, lactoferrin concentration = 8.5 mg/mL and tween 80 = 1.5 mg/mL)

model. The result suggests that the models used in this research were able to identify operating conditions for preparing of lactoferrin nano-liposomes.

Particle size: The p-values were used as a tool to check the significance of every coefficient. The smaller the magnitude of p is, the more significant the corresponding coefficient is. Values of p less than 0.05 indicate that model terms are significant.

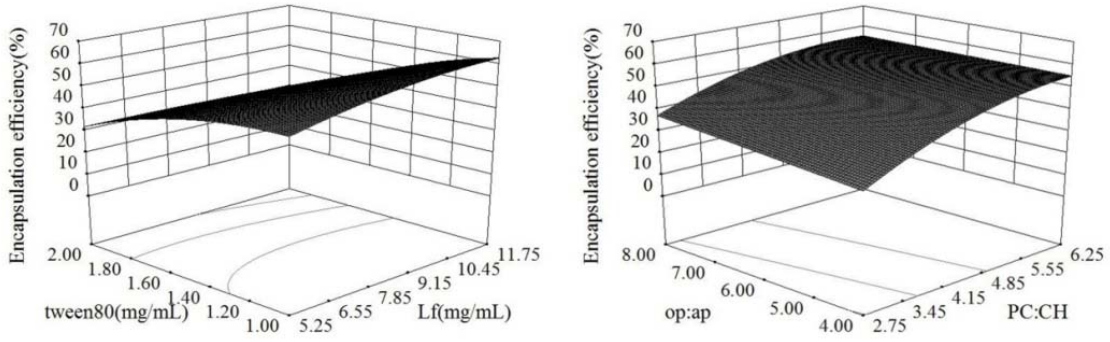
From the model of the particle size, linear effect of all parameters was significant ($p<0.05$). Based on the sum of squares, the importance of the independent variables on yield could be ranked in the following order: organic phase to aqueous phase ratio>lactoferrin concentration> phosphatidylcholine to cholesterol ratio>tween 80. Among the interaction terms, it shows that phosphatidylcholine to cholesterol ratio with lactoferrin concentration and phosphatidylcholine to cholesterol ratio with organic phase to aqueous phase ratio were significant ($p<0.001$, $p<0.05$).

The variation of size with lactoferrin concentration and tween 80 is presented in Fig. 2a. Increasing lactoferrin concentration or tween 80 does not have a significant effect on particle size. Fan and Xu (2007) reported that the size decreases with increasing concentrations of tween 80. This is due to tween 80 has water-soluble chain, making the chemistry of the adjacent liposome down. However, with the surfactant agents increasing, particle size could be also changed. It has also been cited that different drug concentrations have an effect on the particle size and dispersion of the liposome (Zhang *et al.*, 2005). Similar trend has been reported for paclitaxe lmagnetic nanoparticle liposome (Xiao and Wu, 2010), ferrous glycinate nano-liposome (Ding *et al.*, 2011).

The effect of phosphatidylcholine to cholesterol ratio and organic phase to aqueous phase ratio on size is given in Fig. 2b. Wu *et al.* (2007) reported that particle size of papain decreases with decreasing phosphatidylcholine concentration due to that phospholipids constitute the liposome membrane and phosphatidylcholine concentration directly affects the particle size of the liposome.

Encapsulation efficiency: The results in Table 3 showed that the linear effect of phosphatidylcholine to cholesterol ratio, tween 80 and organic phase to aqueous phase ratio were significant ($p<0.05$) whereas lactoferrin concentration is not significant. The effect of independent variables on lactoferrin nano-liposomes is shown in Fig. 3 a, b. increasing lactoferrin concentration increased encapsulation efficiency. At higher lactoferrin concentration, encapsulation efficiency is enhanced due to that more protein was encapsulate into the vesicles. On the other hand, increasing phosphatidylcholine to cholesterol ratio increased encapsulation efficiency. It might due to that cholesterol be able to change the order of mobility of lecithin in the lipid bilayer, thus reinforcing the membrane stability (Niu *et al.*, 2011). Similar results were observed in the studies by Kontogiannopoulos *et al.* (2011) and Xiong *et al.* (2009).

Optimization: Our optimization experiments were designed to find the maximum encapsulation efficiency and minimum particle size of lactoferrin nano-



(a) (b)

Fig. 3**:** Response surface for the effect of independent variables on encapsulation efficiency of lactoferrin nano-liposomes: lactoferrin concentration and tween 80 (a, phosphatidylcholine to cholesterol ratio = 4.5 and organic phase to aqueous phase ratio = 6), and phosphatidylcholine to cholesterol ratio and organic phase to aqueous phase ratio (b, lactoferrin concentration = 8.5 mg/mL and tween 80 = 1.5 mg/mL)

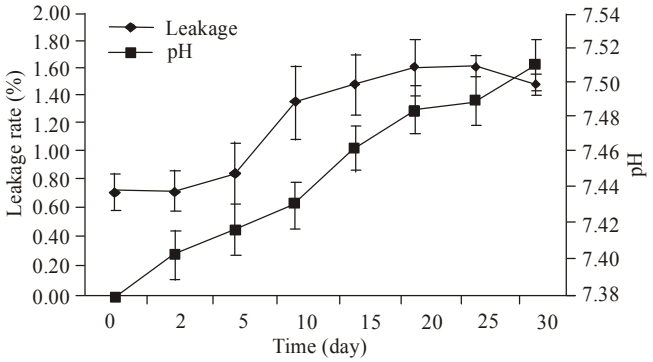


Fig. 4**:** Storage stability of nano-liposomes. Data reported are the mean values±standard variation of three replications

Table 4**:** Predicted optimum conditions of preparation of lactoferrin nano-liposomes

Factor	Low	High	Optimum
Phosphatidylcholine: cholesterol	1	8	5.770
Lactoferrin concentration	2	15	11.71
Tween 80	0.5	2.5	1.180
Organic phase: aqueous phase	2	10	4.590

Table 5**:** Predicted and experimental values of the responses obtained at optimum conditions

Response	Predicted value	Experimental value
PS (nm)	214	220 ±12
EE (%)	60.23	59.80±1.38

liposomes. Table 4 shows phosphatidylcholine/ cholesterol ratio, lactoferrin concentration, tween 80 and organic phase/aqueous phase ratio were selected in the range of 1-8, 2-15, 0.5-2.5 and 2-10 mg/mL respectively. Table 5 shows the conditions given the lowest value of particle size (214) with highest encapsulation efficiency (60.23%) and the predicted values are close to the experimental values. The results indicate that the model used can identify operating conditions for preparing lactoferrin nano-liposomes. These are: phosphatidylcholine to cholesterol ratio of 5.77, lactoferrin concentration of 11.71 mg/mL, tween 80 of 1.18 mg/mL and organic phase to aqueous phase ratio of 4.59.

Stability: The lactoferrin nano-liposomes were subjected to storage stability study for the period of 30 days. The storage stability of lactoferrin nano-liposomes composed of PC: CH ratio of 5.77, lactoferrin concentration of 11.71 mg/mL, tween 80 of 1.18 mg/mL and organic phase to aqueous phase ratio of 4.59 at 4°C and pH 7.4, is presented in Fig. 4. As it shows, pH and the leakage ratio of lactoferrin nano-liposomes tended to increase with increasing storage period. The leakage of lactoferrin nano-liposomes might be attributed to hydroxylation and degradation of bilayer membranes and/or vesicle fusion/aggregation (Flatena *et al.*, 2008; Hincha, 2003).

CONCLUSION

The effect of cholesterol to phosphatidylcholine ratio, lactoferrin concentration, tween 80 and aqueous phase to organic phase ratio on preparing lactoferrin nano-liposome were studied. Second-order polynomial models were obtained for predicting particle size and encapsulation efficiency. While increasing the cholesterol to phosphatidylcholine ratio increased the particle size and encapsulation efficiency. Numerical optimization determined the optimum preparation conditions, which were phosphatidylcholine to cholesterol ratio of 5.77, lactoferrin concentration of 11.71 mg/mL, tween 80 of 1.18 mg/mL and organic phase to aqueous phase ratio of 4.59. Furthermore, pH and leakage ratio of nano-liposome was tested for the period of 30 days. The lactoferrin nano-liposome showed an acceptable stability. Further study is needed to verify in vivo and in food bioavailability of lactoferrin nano-liposomes.

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