I&EC research
Industrial & Engineering Chemistry Research

Article

pubs.acs.org/IECR

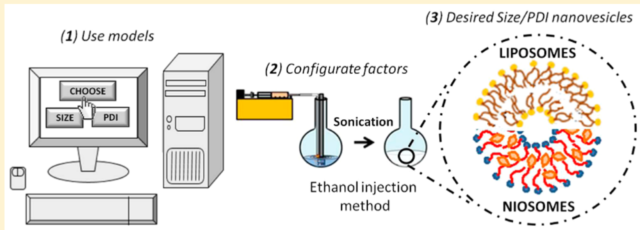
Using Factorial Experimental Design to Prepare Size-Tuned Nanovesicles

Pablo García-Manrique,[†,‡] María Matos,[†] Gemma Gutiérrez,[†] Oscar R. Estupiñán,[†,‡] María Carmen Blanco-Lopez,[‡] and Carmen Pazos*,[†]

[†]Department of Chemical and Environmental Engineering and [‡]Department of Physical and Analytical Chemistry, University of Oviedo, Julián Clavería 8, 33006 Oviedo, Spain

Supporting Information

ABSTRACT: The aim of this work was to prepare size-tuned nanovesicles using a modified ethanol injection method (EIM) by applying factorial experimental design. Stable size-tuned nanovesicles (liposomes and niosomes) with controlled sizes and high EE values for hydrophobic compounds (Sudan Red 7B and vitamin D_3) were achieved. Equations that were able to predict the mean particle sizes, in the ranges of 55−156 nm for liposomes and 224−362 nm for niosomes with PDI values between 0.032 and 0.378, were obtained. These customized soft nanoparticles could be suitable in food, cosmetic, pharmaceutical, or medical applications, such as diagnosis or therapy.



1. INTRODUCTION

Controlled preparation of nanoparticles has attracted great interest in recent years.[1] Nanovesicles are an important family of organic nanoparticles, produced by bottom-up nanotechnology, with relevant applications in biomedicine,[2] food science,[3] analytical chemistry,[4,5] and biosensors.[6] They are considered soft nanoparticles because interactions among their molecular components are similar to those arising from biological systems.[7] Most of the work describing their preparation for specific uses has focused on the optimization of their composition with the aim of maximizing encapsulation efficiency, delivery, or delivery control.

However, size is one of the most critical properties (together with shape and surface chemistry) for understanding cell-uptake processes and, therefore, bioavailability and targetability.[7] Several studies have focused on the optimization of the drug encapsulation efficiency while considering size as just a property for controlling administration parameters, such as penetration kinetics in topical formulations. For example, Padamwar et al.[8] studied the encapsulation of vitamin E in liposomes and found that the amount of lipids yielded a positive correlation with size, which was, in turn, negatively correlated with penetration efficiency into the skin. Sometimes, size has been found to increase with higher amounts of membrane components, such as cholesterol, whereas it decreased with higher amounts of surfactants (e.g., Tween 80). Simultaneously, cholesterol or surfactants can affect encapsulation efficiency (EE). Optimal situations can be reached as a compromise at intermediate levels of both factors. In that case, Taha[9] also reported an interaction between membrane-component concentration and size reduction by ultrasound, making factor optimization an essential task. In other cases, an opposite effect was observed, and higher concentrations of membrane components (such as Span 60 and cholesterol) produced larger sizes and increased EEs. It is useful to deliver efficient amounts of a selected drug into superficial skin layers without systemic absorption.[10] On this basis, the goal of our work was to set up a bulk method for producing nanovesicles of controlled size that could be subsequently modified for specific applications.

Vesicles are colloidal particles in which a concentric bilayer made up of amphiphilic molecules surrounds an aqueous compartment. These vesicles are commonly used to encapsulate both hydrophilic and lipophilic compounds, for food, cosmetic, pharmaceutical, or medical applications, such as diagnosis or therapy.[11] Hydrophilic compounds are entrapped into the aqueous compartments between bilayers, whereas lipophilic compounds are preferentially located inside the bilayers.[12,13] The most common types of vesicles are liposomes and niosomes.

Liposomes were first described by Bangham et al. in 1965,[14] and they are basically spherical bilayer vesicles formed by the self-assembly of phospholipids. This self-assembly process is based on the interactions occurring between phospholipids and water molecules, where the polar head groups of phospholipids are exposed to the aqueous phases (inner and outer) and the hydrophobic hydrocarbon tails are forced to face each other in a bilayer.[15] Because of the presence of both lipid and aqueous phases in liposome structures, they can be used for the encapsulation, delivery, and controlled release of hydrophilic, lipophilic, and amphiphilic compounds.[15,16]

On the other hand, niosomes are vesicles formed by the self-assembly of nonionic surfactants in aqueous media resulting in

Received: April 21, 2016
Revised: July 28, 2016
Accepted: August 10, 2016

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

Table 1. Plackett−Burman Fractional Factorial Design: Responses, Levels, and Factors

response code	meaning
Y_1	*Z*-average size of PC liposomes
Y_2	PDI of PC liposomes

	factors									
	formulation			injection			evaporation		sonication	
level	O/A (X_1)	C (X_2) (g/L)	I (X_3) (mM)	Q_V (X_4) (mL/h)	T_I (X_5) (°C)	N_S (X_6) (rpm)	T_E (X_7) (°C)	N_R (X_8) (rpm)	A (X_9) (%)	t (X_{10}) (min)
−1	5:50	2.5	10	50	30	350	35	30	25	15
1	20:50	6.0	150	215	60	900	60	120	42	30

batch	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	Y_1	Y_2
PB1	1	1	−1	−1	−1	−1	1	−1	1	1	90	0.254
PB2	1	−1	−1	1	−1	1	1	1	1	−1	93	0.129
PB3	−1	−1	−1	−1	−1	−1	1	1	−1	−1	97	0.152
PB4	1	−1	1	−1	1	0	1	−1	1	−1	72	0.205
PB5	−1	1	−1	1	−1	−1	1	−1	−1	1	258	0.413
PB6	1	1	1	1	−1	−1	−1	1	−1	−1	102	0.176
PB7	−1	−1	1	1	−1	−1	−1	−1	1	1	84	0.218
PB8	1	−1	−1	1	1	1	−1	1	−1	1	106	0.240
PB9	−1	−1	−1	−1	1	1	−1	1	1	1	71	0.229
PB10	1	−1	1	−1	−1	−1	−1	−1	−1	1	81	0.141
PB11	−1	1	1	−1	1	−1	1	1	−1	1	152	0.316
PB12	1	1	1	1	1	1	1	1	1	1	65	0.260
PB13	−1	−1	1	1	1	1	1	−1	−1	−1	87	0.189
PB14	−1	1	1	−1	−1	1	−1	1	1	−1	115	0.273
PB15	1	1	−1	−1	1	1	−1	−1	−1	−1	113	0.199
PB16	−1	1	−1	1	1	−1	−1	−1	1	−1	74	0.271

closed bilayer structures.[13,17,18] As liposomes, their formation process is a consequence of unfavorable interactions between surfactants and water molecules, and they can also entrap hydrophilic, lipophilic, and amphiphilic compounds.[19,20]

Niosomes exhibit a number of advantages over liposomes, such as higher stability, easy access to raw materials, lower toxicity, high compatibility with biological systems, non-immunogenicity, and versatility for surface modification.[20]

Cholesterol is commonly used as a membrane additive for nanovesicle preparation to improve vesicle stability, entrapment efficiency, and release under storage.[20] It increases vesicle size and rigidity, improving encapsulation efficiency, but at high concentrations, it can adversely affect the encapsulation rate.[21,22] Cholesterol also plays a fundamental role in niosome formation when hydrophilic surfactants are used (hydrophile/lipophile balance of ∼10).[20]

More than 20 different methods have been identified for nanovesicle preparation, and these methods were recently reviewed.[23,24] In this work, a modified ethanol injection method (EIM) is used, because it offers some advantages over other methods, such as simplicity, absence of potentially harmful chemicals, and suitability for scaleup.[25]

The conventional EIM was first described in 1973.[26] In this technique, lipids/surfactants and additives are first dissolved in an organic solvent, such as diethyl ether or ethanol, and then injected slowly through a syringe into an aqueous phase containing the compound of interest. Then, the organic solvent is removed using a vacuum rotary evaporator. When ethanol is used as the organic solvent, the spontaneous formation of vesicles occurs as soon as the organic solution is in contact with the aqueous phase,[27] but vigorous agitation is needed to obtain narrow size distributions. For this purpose, a final sonication stage was applied in this study after organic-phase removal by vacuum evaporation.

However, a large number of variables are involved in this modified EIM, and selection of the most important of them (screening) is a crucial step in rationally preparing vesicles by this versatile method. In this work, the *Z*-average size and polydispersity index (PDI) were selected as the dependent variables. They are considered to be of great importance in nanovesicle design because most of the final applications of these vesicular systems are directly related to these two parameters. Factorial experimental design and the analysis of variance (ANOVA) methodology are appropriate and efficient statistical tools that permit the effects of several factors that influence responses to be studied by varying the factors simultaneously in a limited number of experiments.

In the recent past, design of experiments (DoE) has been extensively used for the study and optimization of vesicles and other similar organic materials. Different designs can be applied to reduce the number of factors involved in the preparation techniques[28] and, therefore, to minimize the number of experiments without losing valuable information. Plackett−Burman design is a type of fractional design involving relatively few runs,[29] commonly used for the screening of variables.

Another important role of DoE is in the optimization of nanovesicle composition for the enhancement of intended purposes. For instance, it has been applied to the formulation of liposomes (phospholipid and cholesterol ratio) for the topical delivery of vitamin E,[8] hybrid liposomes (with both low- and high-transition-temperature phospholipids) to improve the encapsulation and delivery of silymarin,[30] and niosomes for topical delivery applications.[10,31] DoE has also been used to enhance the transdermal flux of raloxifene hydrochloride[32] and diclofenac diethylamine[33] loaded transfersomes and of other polymeric nanoparticles encapsulating an anticancer drug.[34] Moreover, the interactions between vesicles and proteins, such as pectin, to improve drug-delivery properties has been studied by

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

DoE.[35] Nanostructured lipid carriers (NLCs) loaded with flurbiprofen were also produced under optimal conditions using full factorial design.[36]

In this work, an initial fractional factorial design with two levels (Plackett–Burman) was used to screen the most important factors in vesicle preparation by the EIM. Then, a 2^3 two-level full factorial design using center-point replicates was applied to study the influence of the main factors and their interactions on the Z-average size and PDI. Once the appropriate operating conditions were determined, vesicle stability was studied by using multiple light scattering technology and by measuring the encapsulation efficiencies (EEs) of different compounds.

2. MATERIALS AND METHODS

2.1. Materials. Phosphatidylcholine (PC) (predominant species $C_{42}H_{80}NO_8P$, MW = 775.04 g/mol) from soybean (Phospholipon 90G) was a kind gift from Lipoid (Ludwigshafen, Germany). Sorbitan monostearate (Span 60, S60) ($C_{24}H_{46}O_6$, MW = 430.62 g/mol) and cholesterol (Cho) ($C_{27}H_{46}O$, MW = 386.65 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO). All membrane components were dissolved in absolute ethanol (Sigma-Aldrich, St. Louis, MO).

Methanol, acetonitrile, 2-propanol, and acetic acid of high-performance liquid chromatography (HPLC) grade were supplied by Sigma-Aldrich (St. Louis, MO).

A phosphate buffer (PB) solution (10 mM, pH 7.4) was used in all experiments as the aqueous phase. The buffer solution was prepared in Milli-Q water by dissolving proper amounts of sodium dihydrogen phosphate and sodium hydrogen phosphate, supplied by Panreac (Barcelona, Spain). Sodium chloride from Panreac (Barcelona, Spain) was added to increase the ionic strength when required according to the experiments listed in Table 1. For the encapsulation experiments, Fat Red Bluish or Sudan Red 7B dye ($C_{24}H_{21}N_5$, MW = 379.46 g/mol) and cholecalciferol or vitamin D_3 ($C_{27}H_{44}O$, MW = 384.64 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Factorial Design of Experiments. Factors that could potentially affect the size of vesicles produced by the EIM were classified in four groups, according to the different steps involved in this preparation method: formulation (organic/aqueous phase volume ratio, phospholipid concentration, and ionic strength), injection (injection flow rate, temperature, and stirring speed), evaporation (temperature and rotation speed), and sonication (amplitude and time of sonication).

To identify the relative effects of variables on the response, a two-level fractional factorial design was used. A Plackett–Burman (P–B) resolution III design with n = 16 runs was proposed for screening of the initial factors. Two levels were selected for each variable.

Table 1 lists the factors and levels involved in the P–B fractional factorial design used, where O/A is the organic/aqueous phase volume ratio, C is the concentration of phospholipid, I is the ionic strength, Q_V is the injection flow rate, T_I is the injection temperature, N_S is the stirring speed during injection, T_E is the evaporation temperature, N_E is the evaporator rotation speed, A is the sonication amplitude, and t is the sonication time.

In a second step, a 2^3 full factorial design with center-point repetitions (n = 5) was carried out to study the main effects and interactions between factors previously selected by the screening design (Table 2). All other factors were fixed at certain values.

In both designs, mean diameter (Z-average size) and PDI were selected as response variables. Minitab statistical software

Table 2. Full Factorial Design (2^3) with Center-Point Repetitions (n = 5): Factors, Levels, and Responses

response code	meaning
Y_1	Z-average size of PC liposomes
Y_2	PDI of PC liposomes
Y_3	Z-average size of S60:Cho niosomes
Y_4	PDI of S60:Cho niosomes

level	factors		
	O/A (X_1)	C (X_2) (g/L)	A (X_3) (%)
−1 (low)	5:50	2	30
0 (medium)	12.5:50	5	42.5
1 (high)	20:50	8	55

batch	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
FF1	1	−1	1	65	0.299	305	0.075
FF2	1	1	−1	97	0.249	362	0.136
FF3	−1	1	−1	149	0.296	294	0.206
FF4	−1	1	1	88	0.307	262	0.291
FF5	−1	−1	1	64	0.342	242	0.120
FF6	1	1	−1	100	0.257	360	0.143
FF7	1	1	1	64	0.272	241	0.182
FF8	−1	−1	−1	90	0.196	235	0.078
FF9	0	0	0	82	0.219	301	0.195
FF10	1	−1	−1	84	0.205	253	0.032
FF11	−1	1	1	107	0.297	276	0.235
FF12	−1	1	−1	156	0.308	275	0.145
FF13	1	−1	1	65	0.378	248	0.066
FF14	1	−1	−1	97	0.246	268	0.045
FF15	−1	−1	−1	84	0.173	239	0.094
FF16	0	0	0	75	0.224	305	0.253
FF17	0	0	0	84	0.250	317	0.118
FF18	1	1	1	55	0.307	224	0.203
FF19	0	0	−1	77	0.242	308	0.241
FF20	0	0	0	84	0.251	337	0.171
FF21	−1	−1	1	69	0.343	233	0.124

(version 17) was used for all data analysis. Analysis of variance (ANOVA) was used for this purpose.

Once the models were obtained taking into account significant factors and interactions, a set of selected size-tuned vesicles were prepared and characterized.

2.3. Vesicle Preparation. For liposome preparation, appropriate weighed amounts of PC were dissolved in different volumes of absolute ethanol (5–20 mL range). The same procedure was applied to niosome preparation by weighing and dissolving S60 and Cho in a 1:0.5 weight ratio. Then, the organic solution was injected, with a syringe pump (KD Scientific, Holliston, MA) at a flow rate of 120 mL/h, into Milli-Q water that was kept at 60 °C and stirred at 500 rpm. Once vesicles formed, ethanol was removed at 40 °C under reduced pressure (90 kPa) in a rotary evaporator. The resulting vesicular systems were further sonicated for 15 min (CY-500 sonicator, Optic Ivymen System, Biotech SL, Barcelona, Spain), using and amplitude of 30–55%, a power of 500 W, and a frequency of 20 kHz. The sonication probe was placed in a 100 mL glass beaker at a constant depth, 1.5 cm above the container bottom.

2.4. Vesicle Characterization. *2.4.1. Vesicle Size.* The Z-average size and PDI of vesicles were determined by dynamic light scattering (DSL) using a Zetasizer Nano ZS system (Malvern Instruments Ltd., Malvern, U.K.). Three independent samples were taken from each formulation, and measurements

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

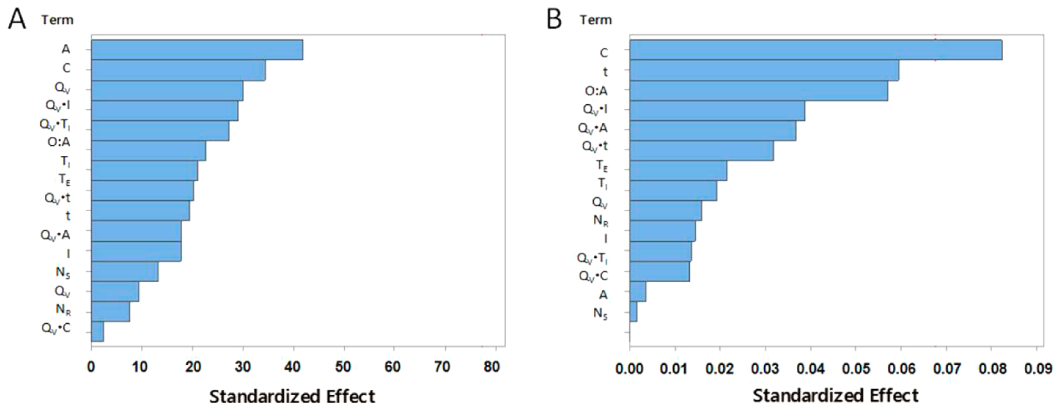


Figure 1. Pareto chart of the standardized effects of independent variables (factors) on the (A) *Z*-average size and (B) PDI of PC liposomes for the Plackett−Burman fractional factorial design.

were performed three times at room temperature without dilution.

2.4.2. Vesicle Morphology. Morphological analysis of vesicles was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II transmission electron microscope (Tokyo, Japan). A sample drop was placed on a carbon-coated copper grid, and excess sample was removed with filter paper. Then, a drop of 2% (w/v) phosphotungstic acid (PTA) solution was applied to the carbon grid and allowed to stand for 1 min. Once the excess staining agent had been removed with filter paper, the sample was air-dried, and the thin film of stained and fixed vesicles was observed with the transmission electron microscope.

2.4.3. Vesicle Stability. Vesicle stability was determined by measuring backscattering (BS) profiles in a Turbiscan Lab Expert apparatus (Formulaction, L'Union, France) provided with an aging station (Formulaction, L'Union, France).

Samples were placed in cylindrical glass test cells, and backscattered light was monitored at 30 °C as a function of time and cell height every 2 h for 7 days.

The optical reading head scans the sample in the cell, providing BS data every 40 μm in percentages relative to standards as a function of the sample height (in millimeters). These profiles build up a macroscopic fingerprint of the sample at a given time, providing useful information about changes in the size distribution or appearance of a creaming layer or a clarification front with time.[3,37,38]

2.4.4. Encapsulation Efficiency (EE). EE also provides useful information related to the stability of the vesicle membrane. Hydrophilic compounds are entrapped in aqueous compartments between bilayers, whereas lipophilic compounds are preferentially located within the surfactant or lipid bilayer.[39] Substances such as drugs, bioactive compounds, dyes, and nanomaterials incorporated into vesicles can also affect the morphology and stability of the final dispersion.

For the purpose of determining EEs, Sudan Red 7B and vitamin D_3 (hydrophobic compounds) were encapsulated in the two different formulations.

Each compound was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) (HP series 1100 chromatograph, Hewlett-Packard, Palo Alto, CA). Before RP-HPLC analysis could be performed, the nonencapsulated compound had to be removed by passing the sample through a Sephadex G-25 column (GE Healthcare Life Sciences, Wauwatosa, WI). Then, both filtered and nonfiltered samples were diluted 1:10 (v/v) with methanol to facilitate vesicle rupture and to extract the encapsulated compound. EE was calculated according to the equation

$$\text{EE (\%)} = \frac{\text{peak area of filtered sample}}{\text{peak area of unfiltered sample}} \times 100 \quad (1)$$

The RP-HPLC system was equipped with an HP G1315A UV/vis absorbance detector (Agilent Technologies, Palo Alto, CA). The column was a Zorbax Eclipse Plus C18 column with a particle size of 5 μm, 4.6 mm × 150 mm (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of a mixture of (A) 100% Milli-Q-water and (B) 100% methanol with gradient elution at 0.8 mL/min. The step gradient started with a mobile phase of 80% A, running 100% mobile phase B starting in minute 5 for 10 min. Mobile phase B was fed for 2 min after each injection to prepare the column for the next sample. The separation was carried out at 30 °C. Different wavelengths were used for the UV/vis detector, namely, 533 nm for Sudan Red 7B and 270 nm for vitamin D_3.

3. RESULTS AND DISCUSSION

3.1. Effects of Variables on Morphological Characteristics. The responses (*Z*-average size and PDI) of each batch from P−B design were measured by dynamic light scattering (DLS). The relative importance of the main effects on the *Z*-average size and PDI of PC liposomes are shown in the Pareto chart given in Figure 1.

Researchers must be aware of the confusion of main effects with two-factor interactions in this type of design (resolution III), where the alias structure is too complex. However, we decided to use the initial Plackett−Burman design only for screening purposes and selection of the main factors from the Pareto chart, as is usually accepted. Effects were selected by applying the hierarchical ordering principle, known sometimes as the sparsity-of-effects principle, where higher-order effects (three- or four-way interactions) are sacrificed to study lower-order effects (main effects first and two-way interactions next). This principle suggests that priority should be given to the estimation of lower-order effects, especially when resources (time and money) are scarce. This postulate is an empirical principle whose validity has been confirmed by the analysis of many real experiments.

According to these data, the most important variables for both responses are the organic/aqueous phase volume ratio, the (final aqueous-phase) phospholipid concentration, and the sonication amplitude. These results are in good agreement with previous studies carried out by Kremer et al.,[40] who evaluated the effects of

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

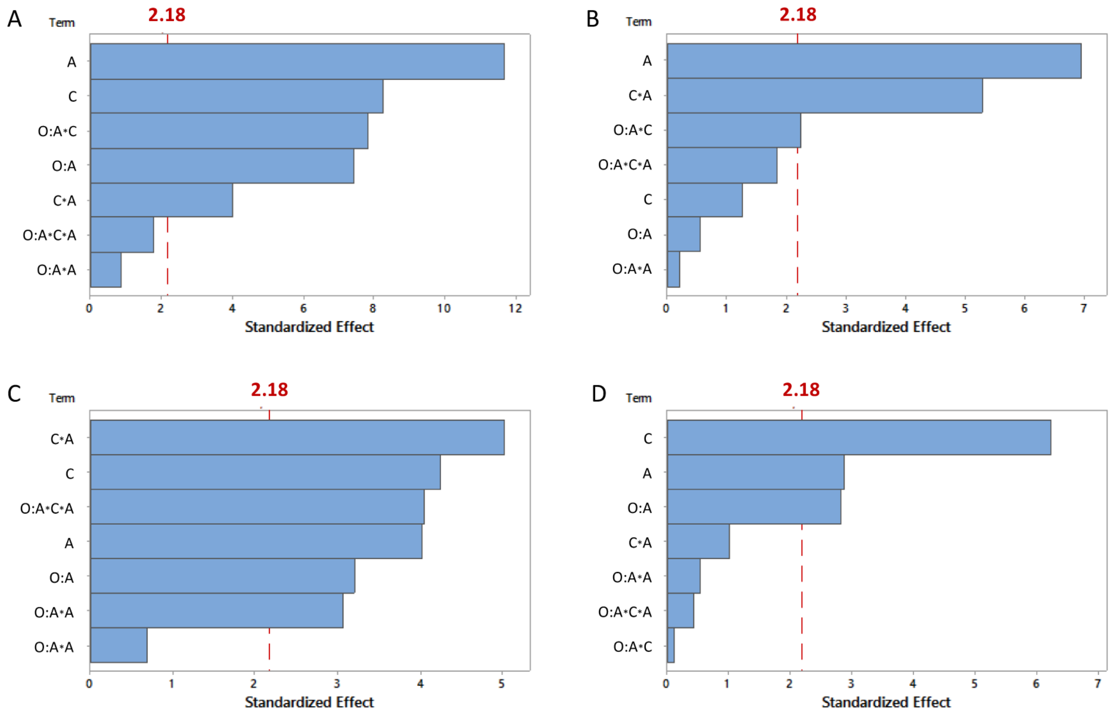


Figure 2. Pareto chart of the standardized effects of independent variables (factors) on the (A,C) *Z*-average size and (B,D) PDI of (A,B) PC liposomes and (C,D) S60:Cho niosomes (1:0.5, w/w) for the 2^3 full factorial design.

some preparation variables on the size and polydispersity of liposomes made from two different natural phosphatidylcholines. Their experimental results showed that the most important factor in the final size of the liposomes was the lipid concentration in the alcohol injected into the buffer solution. This factor corresponds to the interaction of the lipid amount and the flow rate of organic solvent injected, two factors present in the Pareto chart in Figure 1. The same explanation was postulated by other authors,[8,41,42] confirming that the lipid concentration clearly affects the liposome size. This factor was found to be the most relevant one for controlling morphological characteristics of phosphatidylcholine liposomes. Szoka[43] found that stirring, ionic strength, and temperature of the aqueous phase could also contribute to the final size, but the effects of these factors were smaller than those observed for lipid concentration, organic/aqueous phase ratio, and chemical nature of the organic solvent (a parameter not included in our study). Therefore, the experimental results in Figure 1 confirm the previously reported observations.[43]

The ethanol injection method is usually chosen because it avoids the sonication step, which is needed in several other methods of liposome preparation, such as the thin-film hydration method. Preliminary experiments (data not shown) indicated that sonication is a crucial step for reducing the size of both liposomes and niosomes. Alternatively, small vesicles can be produced without sonication by using low concentrations of lipids/surfactants, but with low yield. This is why we decided to include this step as a factor in the present study.

3.2. PC Liposomes. The first three main effects from the Pareto chart obtained for the P−B design were selected for the 2^3 full factorial design. The ANOVA results for *Z*-average size and PDI values are listed in Tables S1 and S2 (Supporting Information), respectively, whereas the corresponding Pareto chart is shown in Figure 2. Mean sizes in the range of 55−156 nm with PDI values between 0.173 and 0.378 were obtained for PC liposomes (with standard deviations ranging from 0.304 to 4.40 nm for size and from 0.003 to 0.053 for PDI). Similar size ranges were also obtained using the EIM in other previously reported studies.[22,27,41,43,44]

The normality, variance homogeneity, and randomness assumptions were tested with a normal probability plot, frequency histogram, and residuals versus fits and residuals versus order plots, respectively (Supporting Information, Figure S2).

No clear aberrant tendencies were observed, because the residuals tended to form a line, no typical cornet pattern was observed, and no time-based pattern was detected. Only some outlier values were detected (Cook's distance and DFITS values are given in Table S3 of the Supporting Information).

The ANOVA results allowed for an analysis of the contributions of the effects of the independent variables on the response function (mean size of PC liposomes). In this case, significant two-way interactions were identified: $(O/A) \times C$ and $C \times A$ (see Figure 3). Larger sizes are reached when the organic solution has a higher lipid concentration (more than 20 g/L). On the other hand, the $C \times A$ interaction reveals that the degree of size reduction upon application of a higher amplitude depends on the total lipid concentration present in the medium (referred to the final volume of the dispersion).

All of the main effects are significant ($p < 0.05$), with a positive effect on the mean size (a higher response value with an increase in the factor level) for the total lipid concentration and a negative effect (a lower response value with a decrease in factor level) for the organic/aqueous phase volume ratio and the sonication amplitude.

These effects can be explained according to a previously reported vesicle formation model.[45−47] This model relies on the formation of vesicles through intermediate structures, such as phospholipid bilayer fragments and sheet-like micelles. These

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

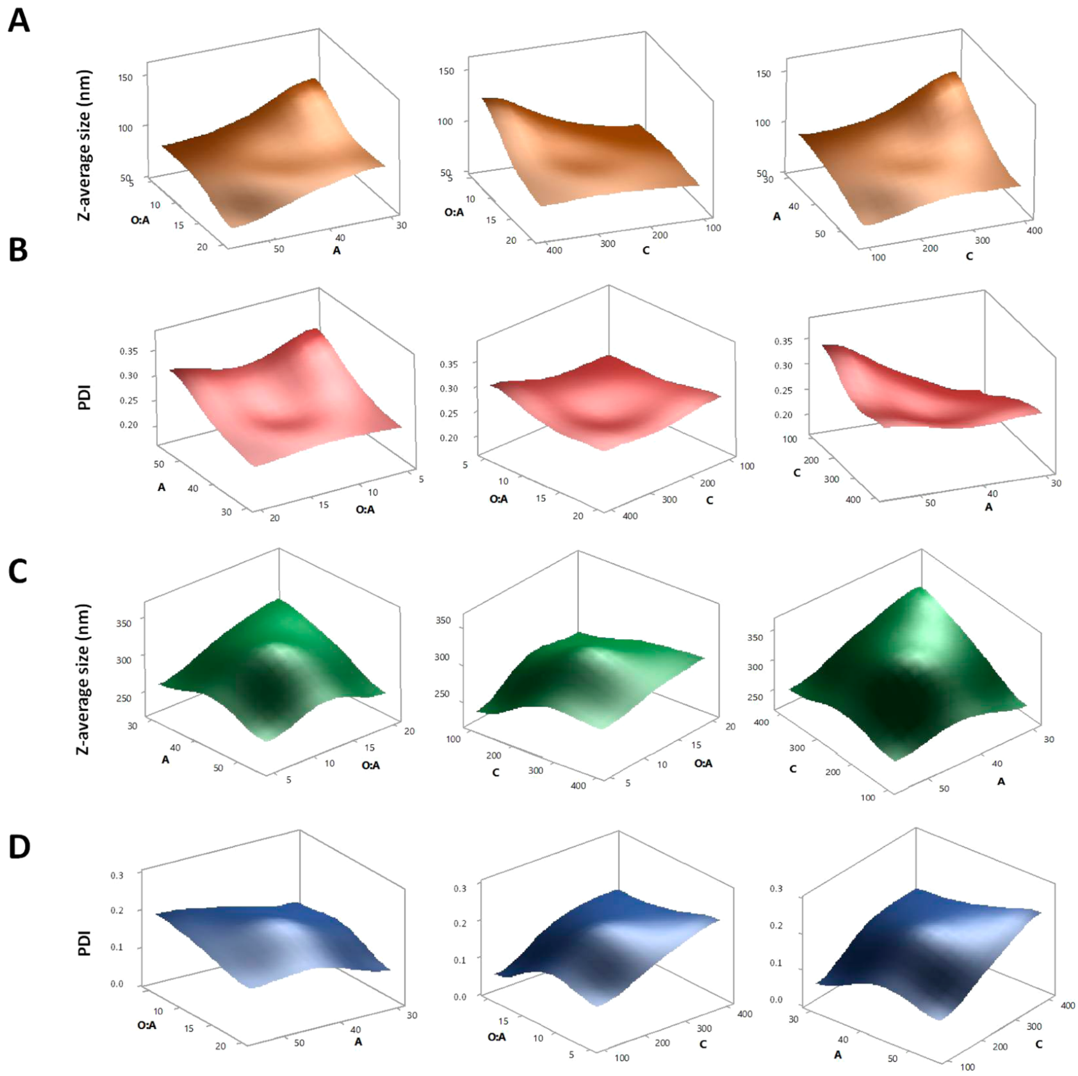


Figure 3. Three-dimensional (3D) response surface plots for the factors O/A (organic/aqueous phase volume ratio), C (lipid or surfactant/stabilizer concentration, g/L), and A (sonication amplitude, %) for the (A,C) Z-average size and (B,D) PDI of (A,B) PC liposomes and (C,D) S60:Cho niosomes (1:0.5, w/w).

intermediates are the result of amphiphilic self-assembly because of their characteristic physicochemical properties.[48]

During the injection of ethanol droplets into the aqueous phase, lipid reorganization inside these dispersed droplets to form bilayers is favored by the fact that lipids energetically prefer a parallel molecular arrangement.[45] These planar structures give rise to closed vesicles when their size induces enough surface tension to close the structure and minimize the bending energy.

The sizes of these intermediates depend directly on the number of lipid molecules (concentration) and the dispersion degree (solubilization) in the organic phase. It is obvious from the previous assessment that higher concentrations of lipids in the droplets will form higher membrane fragments, as our experimental results and previous observations confirm.[8,40−42]

It is also important to know how easily lipid droplets are dispersed, as well as their size and homogeneity. Lipids of higher solubility will then form smaller lipid droplets and, consequently, shorter membrane fragments (and ultimately tiny vesicles).[40] This explains, in a simplified way, why higher organic/aqueous phase ratios yield smaller liposomes.

The negative effect of the sonication amplitude is explained by vesicle rupture, which takes place when an excess of energy is applied to vesicles during the sonication process as a result of the effect of induced cavitation.[49,50] The final effect of ultrasounds can be controlled by varying the input power, ultrasound frequency, sonication time, and probe depth into the container. As frequency increases, liposomes of smaller size are produced as a result of stronger acoustic cavitation events. This assumption was confirmed by our results, in accordance with previous studies.[49,50] It is important to point out that, to minimize the effects of variations in the probe depth, this factor was kept constant at 1.5 cm above the container bottom.

Another aspect to be taken into consideration is the effect of sonication time. It was reported by Silva et al.[49] that sonication time plays an important role in decreasing vesicle size, although they observed that this effect reached a plateau at about 21 min. Our P−B design revealed a positive effect of sonication time on the *Z*-average size (from 15 to 30 min), although it was weaker than the effects of the other variables selected for the 2^3 full factorial design (especially sonication amplitude). A similar influence was observed for the PDI response, but with a stronger effect. We preferred to select sonication amplitude instead of sonication time because one of the goals of controlling factors is to obtain a narrow size distribution.

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

Table 3. Estimated Coded Coefficients for the Considered Effects on the Z-Average Size and PDI of PC Liposomes and S60:Cho Niosomes (1:0.5, w/w)

response	coefficients[a]								
	constant	X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$	R^2
				Z-Average Size					
liposome (Y_1)	89.68	−11.14	12.40	−17.50	−11.75	–	−5.97	–	96.69
niosome (Y_3)	269.82	12.72	16.87	−15.94	–	−12.15	−19.92	−16.04	91.27
				PDI					
liposome (Y_2)	0.280	–	–	0.038	−0.012	–	−0.029	–	89.35
niosome (Y_4)	0.136	−0.026	0.057	0.026	–	–	–	–	84.73

[a] X_1, organic/aqueous phase volume ratio; X_2, PC or S60:Cho concentration (g/L); X_3, sonication amplitude (%).

As the design included a center point with several repetitions ($n = 5$), the presence of curvature in the response variables could be tested (Figure 3). Because curvature seemed to be significant ($p < 0.05$), a term involving center point (Ct Pt) was included in the equations for its estimation.

With all of this information about the effects and their estimated coefficients, the following equation ($R^2 = 96.69\%$) for the *Z*-average size value of PC liposomes (Y_1) was generated

$$Y_1 = 62.8 + 2.55(\mathrm{O/A}) + 0.449C - 0.185A - 0.0185(\mathrm{O/A}) \times C - 0.00555C \times A - 9.26(\mathrm{Ct\ Pt}) \quad (2)$$

Different behavior was observed regarding PDI, which was strongly affected by the sonication amplitude as the only significant main effect and its interaction with the total lipid amount. The O/A × C interaction was also detected, but with a lower effect on the PDI response.

To understand the *C* × *A* interaction, it is important to take into account the effect of the sonication amplitude as the main effect. An increase in this factor leads to a less monodisperse size distribution, that is, higher PDI values. However, according to the interaction, this response depends highly on the total amount of lipids present in the sample. At a low level of the lipid amount, the reduction in size is more effective (as previously mentioned), but the size distribution is large. However, at a high level of the lipid amount, this enlargement of the size distribution is significantly lower.

Curvature in the response was also tested, again revealing a significant presence ($p < 0.05$). For the PDI response (Y_2), the following equation with an R^2 value of 89.35% was obtained

$$Y_2 = -0.160 + 0.00939A - 0.0000420(\mathrm{O/A}) \times C - 0.0000250C \times A - 0.0425(\mathrm{Ct\ Pt}) \quad (3)$$

These equations were formulated with uncoded coefficients, making it easier to use them to predict selected target size and PDI values.

3.3. S60:Cho Niosomes. To investigate whether the selected factors in the P−B design for PC liposomes (a reference model for vesicular systems) produced similar effects with other different formulations, the same 2^3 full factorial design using center-point replicates was performed for a typical niosome formulation, in this particular case, S60:Cho niosomes (1:0.5, w/w). The main variables were the organic/aqueous phase volume ratio (O/A), the total concentration of surfactant and stabilizer (*C*), and the sonication amplitude (*A*).

The ANOVA results for *Z*-average size and PDI values are listed in Table S1 (Supporting Information), and the corresponding Pareto chart and three-dimensional surface plot are shown in Figures 2 and 3, respectively. Mean sizes in the range of 224−362 nm with PDI values between 0.032 and 0.291 were obtained for S60:Cho niosomes (with standard deviations ranging from 1.05 to 7.28 nm for size and from 0.009 to 0.052 for PDI). Similar size and PDI ranges were reported for niosomes prepared by the EIM using Span 60 as the membrane component.[17]

Two-way interactions (O/A × *A*, *C* × *A*) and a three-way interaction (O/A × *C* × *A*) were detected, with sonication amplitude (*A*) as the common factor in these interactions (see Figure 2C). Therefore, it can be postulated that sonication amplitude is the key factor in the niosome size response. The response depends on both the O/A and *C* factor levels, with a higher interaction between the sonication amplitude and the total amount of membrane components. Differences in the magnitude of the coefficient of this factor between liposomes and niosomes can be attributed to the initial size before sonication (smaller for liposomes) and vesicle stability.[50]

The three main effects are significant, but in contrast to the case for liposomes, the organic/aqueous phase volume ratio (O/A) shows a positive effect on niosome size. This behavior could be due to different molecular features of the surfactant and stabilizer that result in different interactions with the organic phase and, therefore, poor or insufficient solubility.

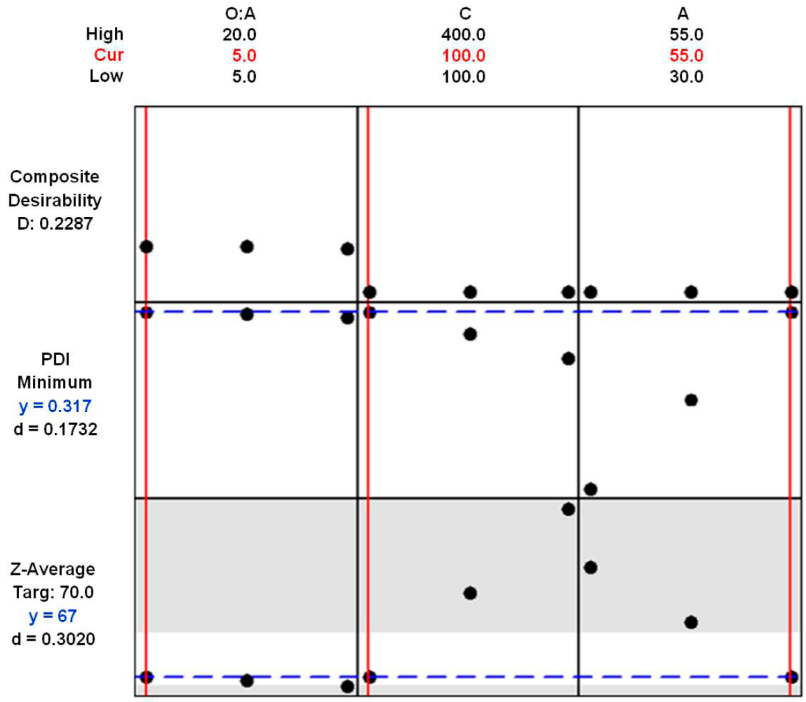
The other two variables (*C*, *A*) have effects similar to those described above for liposomes. Therefore, the same explanation regarding surfactant concentration and sonication amplitude can be applied here to justify their effects on niosome size. In this case, the stronger effect of *C* is explained by the influence of cholesterol on the final size of vesicles, as reported by Padamwar and Pokharkar.[8]

Once again, curvature was detected for the *Z*-average size response. The following equation was obtained to model this case, with an adjusted correlation coefficient (R^2) of 91.27%

$$Y_3 = 236.9 - 4.31(\mathrm{O/A}) - 0.012C - 0.56A + 0.0461(\mathrm{O/A}) \times C + 0.00363C \times A - 0.00114(\mathrm{O/A}) \times C \times A + 44.00(\mathrm{Ct\ Pt}) \quad (4)$$

On the other hand, a completely different behavior was observed regarding the PDI response. Only the three main effects (O/A, *C*, *A*) were found to be significant, and no interactions were found. Two positive effects on the niosome PDI were detected: surfactant/stabilizer concentration and sonication amplitude. In this case, the total concentration of membrane components seemed to have an important role in the vesicle size distribution, as can be seen in the correspondent Pareto chart (Figure 2). This observation once again can be attributed to the solubilization of membrane components in the organic phase. Higher concen-

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX



Parameters

Response	Goal	Lower	Target	Upper	Weight	Importance
Z-Average PC-Liposomes	Target	65	70.000	75.000	1	1
PDI PC-Liposomes	Minimum		0.173	0.378	1	1

Solution

Solution	O:A	C	A	Z-Average PC-Liposomes Fit	PDI PC-Liposomes Fit	Composite Desirability
1	5	100	55	67	0.317	0.2287

Response	Fit	SE Fit	95% CI	95% PI
Z-Average PC-Liposomes	67	4	(57, 76)	(51, 83)
PDI PC-Liposomes	0.317	0.013	(0.308, 0.377)	(0.284, 0.402)

Figure 4. Optimization plot and values of individual (d) and composite (D) desirability provided by the response optimizer (Minitab, version 17) for an example of size-tuned PC liposome (desired size = 70 nm, with a minimum PDI value).

trations of these components require better solubilization in dispersed droplets to reach small membrane fragments.

It is important to note that some combinations of factors yielded narrow size distributions, namely, PDI ≤ 0.100, a value frequently obtained by other preparation methods, such as microfluidic hydrodynamic focusing[51] also using S60:Cho as the formulation.

A negative effect was detected for the organic/aqueous phase volume ratio (O/A). As the final concentration of ethanol increased during the injection process, a smaller size distribution was obtained. As previously mentioned, no interaction between this factor and the total concentration of membrane components was observed.

The following equation with an R^2 value of 84.73% was obtained for the niosome PDI model response (Y_4)

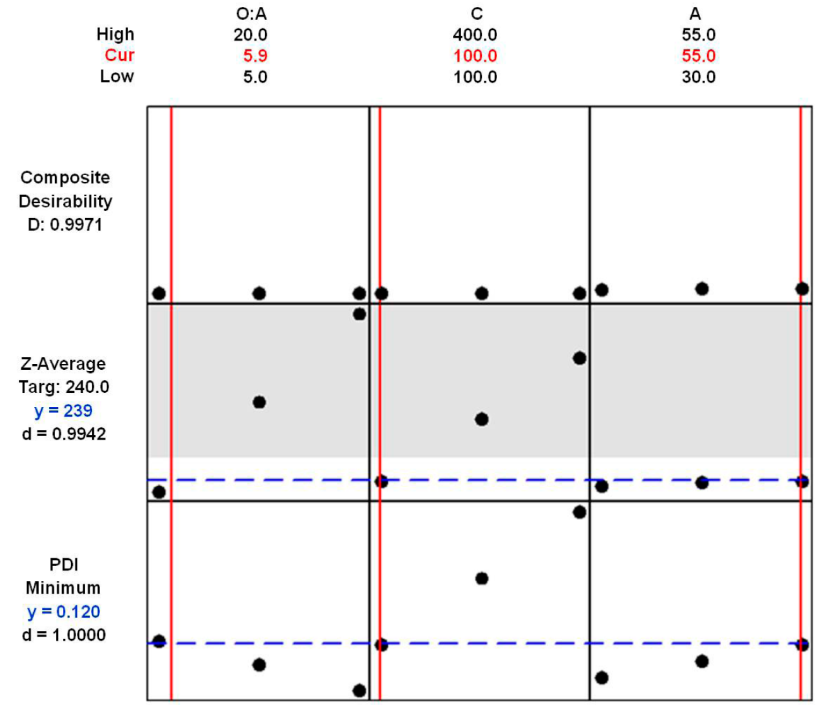
$$Y_4 = 0.053 - 0.00392(\mathrm{O/A}) + 0.000039C + 0.00067A + 0.0597(\mathrm{Ct\,Pt}) \quad (5)$$

The estimated coded coefficients for the considered effects on the Z-average sizes and PDIs of PC liposomes and S60:Cho niosomes are listed in Table 3, as a summary of the factors' influence. Coded coefficients were used to maintain the orthogonality of the designs and to allow for a direct comparison between coefficients.

3.4. Vesicle Characterization. Size-tuned vesicles were prepared under selected operating conditions by applying the models obtained from the experimental design (eqs 2−5) and the assistance of the response optimizer and response predictor in Minitab statistical software (version 17). These tools can be applied to the simultaneous optimization of several responses only when the same set of factors are studied separately, because a common experimental region is needed.

The operating conditions were selected to prepare PC liposomes with a mean size of 70 nm and the minimum PDI value (predicted values of Y_1 = 67 ± 4 and Y_2 = 0.317 ± 0.013) and S60-Cho niosomes with a mean size of 240 nm and the

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX




```
Parameters

Response                     Goal      Lower   Target     Upper   Weight
Importance
Z-Average S60:Cho Niosomes   Target      235   240.000   245.000       1
1
PDI S60:Cho Niosomes         Minimum             0.120     0.291       1
1

Solutions

                                               Z-Average
                              PDI S60:Cho     S60:Cho
                                 Niosomes    Niosomes      Composite
Solution   O:A   C     A            Fit         Fit   Desirability
1          5.9   100   55         0.120         239         0.9971


Response                        Fit   SE Fit       95% CI              95% PI
Z-Average S60:Cho Niosomes      239       11   (  217,    263)     (  198,    281)
PDI S60:Cho Niosomes          0.120    0.025   (0.066, 0.172)      (0.024, 0.214)
```

Figure 5. Optimization plot and values of individual (d) and composite (D) desirability provided by the response optimizer (Minitab, version 17) for an example of size-tuned S60:Cho niosome (1:0.5 w/w) (desired size = 240 nm, with a minimum PDI).

minimum PDI value (predicted values of $Y_3 = 239 \pm 11$ and $Y_4 = 0.120 \pm 0.025$). These sizes and PDI values were selected only as an example. The factor output values were O/A = 5:50, C = 2 g/L, and A = 55% for the liposomes and O/A = 5.9:50, C = 2 g/L, and A = 55% for the niosomes. Figures 4 and 5 show optimization plots and values of individual and composite desirability for size-tuned liposomes and size-tuned niosomes, respectively.

The experimental results showed that the models obtained with the experimental design were accurate, because mean sizes of 69 ± 0.5 nm (PDI = 0.245 ± 0.005) and 233 ± 3 nm (PDI = 0.112 ± 0.004) were obtained for the PC liposomes and S60:Cho niosomes, respectively. The relative error was low for the experimental results regarding mean size (3% for Y_1 and Y_3) but higher for the size distributions (22% for Y_2 and 7% for Y_4).

The sizes and morphologies of the vesicles were investigated by TEM, using a negative contrast. Figure 6 shows black-stained vesicles, as a result of the interactions of the electron beam with PTA, which produces a selective deposit of metal ions that enhances morphological details. The micrographs show spherical structures of approximately 80 nm for the liposomes (Figure 6C) and about 250 nm for the niosomes (Figure 6D). These values agree with the DLS measurements.

Figure 6D shows clusters of niosomes that are all similar in size. Aggregation arose during the drying step prior to TEM measurements, because no flocculation phenomena were monitored with the Turbiscan apparatus.

Slight differences were noticed in the zeta potential measurements, exhibiting low values for both types of vesicles. Niosomes had values of about −16.8 ± 0.7 mV, whereas the liposomes had values of −6.9 ± 0.3 mV. This small value for the liposomes could be due to neutralization of the negative charge from the

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

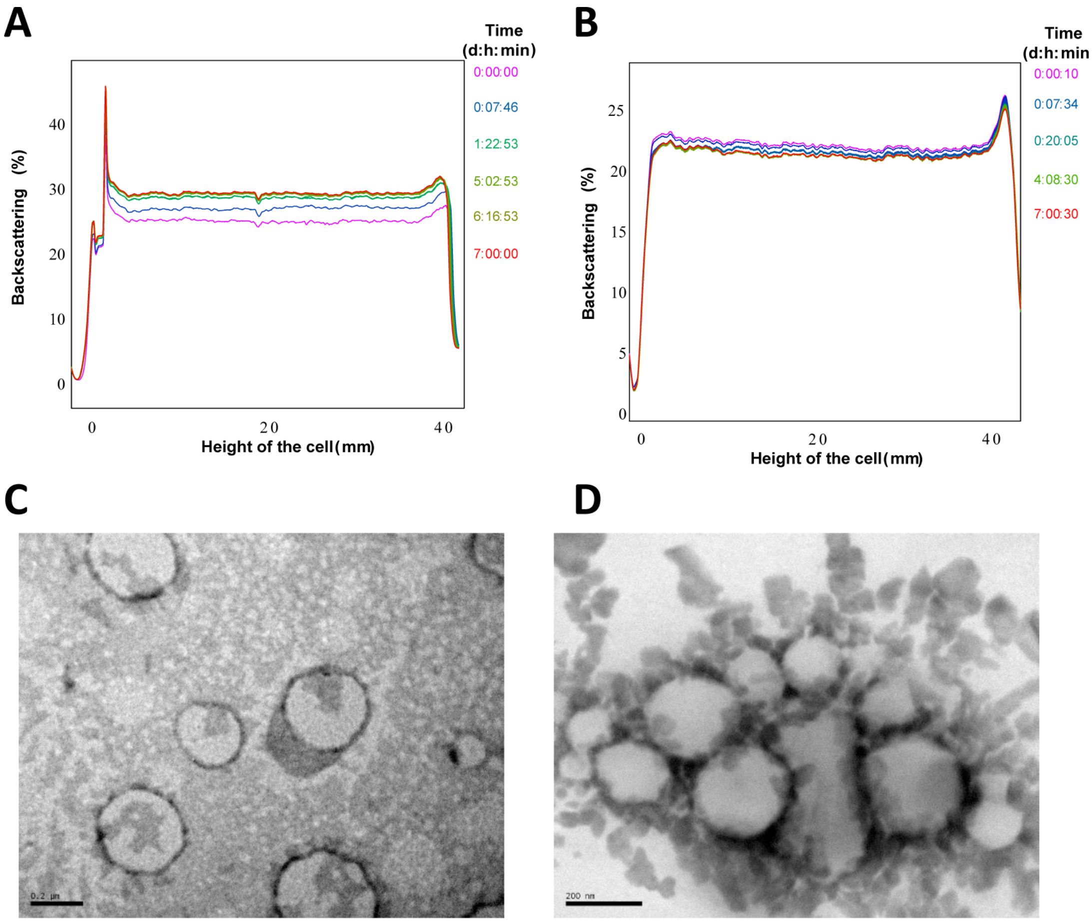


Figure 6. (A,B) BS profiles and (C,D) TEM micrographs of empty vesicles designed with a controlled size and PDI values by applying the models obtained from experimental design: (A,C) PC liposomes and (B,D) S60:Cho niosomes (1:0.5, w/w).

phosphate groups by sodium cations present in the medium (from sodium chloride in the PB solution).

The formulated vesicles exhibited a high stability after 1 week of monitoring time. BS profiles obtained for the PC liposomes are given in Figure 6, where a variation of 4.5% in the middle part of the cell (from 10 to 30 mm) is noticed. A simultaneous slight clarification process was observed in the middle and top parts of the cell in the corresponding transmission profile (results not shown). This was promoted by some movement of the PC liposomes toward the bottom of the cell, resulting in a slight increase in BS (sedimentation). However, this was a reversible process, caused by differences in concentration, with the sample remaining stable and maintaining its initial properties (size and PDI). The vesicles were again characterized after gentle agitation of the cell at the end of the monitoring time with analogous results.

For the S60:Cho niosomes (Figure 6B), the BS profile remained nearly constant (variations of approximately 0.5%) with time, showing high stability. Some variation was also observed in the transmission profile all along the cell height, because the sample was not translucent.

3.4.1. Encapsulation Efficiency (EE). Vesicles containing Sudan Red 7B and vitamin D_3 as model compounds (both lipophilic) were also prepared and characterized. No differences were observed regarding mean size and PDI values or TEM, zeta potential, or Turbiscan measurements, meaning that the entrapped compounds did not affect the vesicle's behavior.

High EE values were obtained for both Sudan Red 7B and vitamin D_3, as expected taking into account their hydrophobic character. EE values up to 90.1% and 88.0% were obtained for Sudan Red 7B encapsulated in PC liposomes and S60:Cho niosomes, respectively. Experiments carried out with vitamin D_3 led to EE values of 99.2% for PC liposomes and 73.9% for S60:Cho niosomes. These results are in good agreement with those of previous studies, where compounds with similar chemical properties were encapsulated.[12,13,27]

4. CONCLUSIONS

In this work, an adequate approximation using DoE was applied to study the influence of experimental factors of the EIM on the mean size and size distribution of PC liposomes and S60:Cho niosomes (1:0.5, w/w).

An initial screening design enabled a reduction of the number of variables. This was a necessary step before carrying out a full factorial design. Finally, response models were applied to prepare selected size-tuned nanovesicles, which were characterized from a stability point of view.

This was achieved with a low number of experiments (58 runs). This methodology enabled two different formulations (liposomes and niosomes, the most common types of nano-vesicles) to be studied in a comparative way. Stable liposomes and niosomes of the targeted sizes were successfully prepared with the model equations obtained, with encapsulation efficiencies higher than 73.9% in all cases for selected hydrophobic compounds.

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX

The most important variables identified by ANOVA were the organic/aqueous phase volume ratio, the (final aqueous-phase) phospholipid concentration, and the sonication amplitude.

These results offer new insights into the mechanism and effects of the factors involved in nanovesicle preparation by the EIM, one of the most easily scaled-up methods for preparing vesicles for several fields of interest.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.iecr.6b01552.

ANOVA results for *Z*-average size and PDI of PC liposomes for the 2^3 full factorial design; Cook's distances and DFITS values for each response in the full factorial designs; optimization contour plot for the factors studied in the full factorial design for both responses; testing for normality, variance homogeneity, and randomness assumptions of ANOVA for the full factorial design (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +34 985103509. E-mail: cpazos@uniovi.es.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Ministerio de Economía y Competitividad (MINECO, Spain), under Grant MINECO-13-CTQ2013-47396-R. This study was also cofinanced by the Consejería de Educación y Ciencia del Principado de Asturias (ref FC-04-COF-50-MEC, PCTI Asturias 2006-2009, ref EQP06-024, and FC15-GRUPIN14-022). We especially thank Prof. Antonia Salas (University of Oviedo) for her advice with the statistical work.

■ REFERENCES

(1) Capretto, L.; Carugo, D.; Mazzitelli, S.; Nastruzzi, C.; Zhang, X. Microfluidic and lab-on-a-chip preparation routes for organic nano-particles and vesicular systems for nanomedicine applications. *Adv. Drug Delivery Rev.* **2013**, *65*, 1496−1532.

(2) Rongen, H. A. H.; Bult, A.; van Bennekom, W. P. Liposomes and immunoassays. *J. Immunol. Methods* **1997**, *204*, 105−133.

(3) Pando, D.; Gutiérrez, G.; Coca, J.; Pazos, C. Preparation and characterization of niosomes containing resveratrol. *J. Food Eng.* **2013**, *117*, 227−234.

(4) Gómez-Hens, A.; Fernández-Romero, J. M. The role of liposomes in analytical processes. *TrAC, Trends Anal. Chem.* **2005**, *24*, 9−19.

(5) Edwards, K. A.; Bolduc, O. R.; Baeumner, A. J. Miniaturized bioanalytical systems: enhanced performance through liposomes. *Curr. Opin. Chem. Biol.* **2012**, *16*, 444−452.

(6) Liu, Q.; Boyd, B. J. Liposomes in biosensors. *Analyst* **2013**, *138*, 391−409.

(7) Canton, I.; Battaglia, G. Endocytosis at the nanoscale. *Chem. Soc. Rev.* **2012**, *41*, 2718−39.

(8) Padamwar, M. N.; Pokharkar, V. B. Development of vitamin loaded topical liposomal formulation using factorial design approach: Drug deposition and stability. *Int. J. Pharm.* **2006**, *320*, 37−44.

(9) Taha, E. I. Lipid vesicular systems: formulation optimization and ex vivo comparative study. *J. Mol. Liq.* **2014**, *196*, 211−216.

(10) Abdelbary, A. A.; AbouGhaly, M. H. H. Design and optimization of topical methotrexate loaded niosomes for enhanced management of psoriasis: Application of Box−Behnken design, in-vitro evaluation and in-vivo skin deposition study. *Int. J. Pharm.* **2015**, *485*, 235−243.

(11) Jadhav, S. M.; Morey, P.; Karpe, M.; Kadam, V. Novel vesicular system: An overview. *J. Appl. Pharm. Sci.* **2012**, *02*, 193−202.

(12) Rajera, R.; Nagpal, K.; Singh, S. K.; Mishra, D. N. Niosomes: a controlled and novel drug delivery system. *Biol. Pharm. Bull.* **2011**, *34*, 945−53.

(13) Uchegbu, I. F.; Vyas, S. P. Non-ionic surfactant based vesicles (niosomes) in drug delivery. *Int. J. Pharm.* **1998**, *172*, 33−70.

(14) Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **1965**, *13*, 238−252.

(15) da Silva Malheiros, P.; Daroit, D. J.; Brandelli, A. Food applications of liposome-encapsulated antimicrobial peptides. *Trends Food Sci. Technol.* **2010**, *21*, 284−292.

(16) du Plessis, J.; Weiner, N.; Müller, D. G. The influence of in vivo treatment of skin with liposomes on the topical absorption of a hydrophilic and a hydrophobic drug in vitro. *Int. J. Pharm.* **1994**, *103*, R1−R5.

(17) Manconi, M.; Sinico, C.; Valenti, D.; Loy, G.; Fadda, A. M. Niosomes as carriers for tretinoin I: Preparation and properties. *Int. J. Pharm.* **2002**, *234*, 237−248.

(18) Manca, M. L.; Manconi, M.; Nacher, A.; Carbone, C.; Valenti, D.; Maccioni, A. M.; Sinico, C.; Fadda, A. M. Development of novel diolein−niosomes for cutaneous delivery of tretinoin: Influence of formulation and in vitro assessment. *Int. J. Pharm.* **2014**, *477*, 176−186.

(19) Mahale, N. B.; Thakkar, P. D.; Mali, R. G.; Walunj, D. R.; Chaudhari, S. R. Niosomes: Novel sustained release nonionic stable vesicular systems — An overview. *Adv. Colloid Interface Sci.* **2012**, *183*−*184*, 46−54.

(20) Moghassemi, S.; Hadjizadeh, A. Nano-niosomes as nanoscale drug delivery systems: An illustrated review. *J. Controlled Release* **2014**, *185*, 22−36.

(21) Mali, N.; Darandale, S.; Vavia, P. Niosomes as a vesicular carrier for topical administration of minoxidil: formulation and in vitro assessment. *Drug Delivery Transl. Res.* **2013**, *3*, 587−592.

(22) Fan, M.; Xu, S.; Xia, S.; Zhang, X. Preparation of salidroside nano-liposomes by ethanol injection method and in vitro release study. *Eur. Food Res. Technol.* **2008**, *227*, 167−174.

(23) Marianecci, C.; Di Marzio, L.; Rinaldi, F.; Celia, C.; Paolino, D.; Alhaique, F.; Esposito, S.; Carafa, M. Niosomes from 80s to present: The state of the art. *Adv. Colloid Interface Sci.* **2014**, *205*, 187−206.

(24) Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: classification, preparation, and applications. *Nanoscale Res. Lett.* **2013**, *8*, 102.

(25) Justo, O. R.; Moraes, Â. M. Analysis of process parameters on the characteristics of liposomes prepared by ethanol injection with a view to process scale-up: Effect of temperature and batch volume. *Chem. Eng. Res. Des.* **2011**, *89*, 785−792.

(26) Batzri, S.; Korn, E. D. Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta, Biomembr.* **1973**, *298*, 1015−1019.

(27) Pham, T. T.; Jaafar-Maalej, C.; Charcosset, C.; Fessi, H. Liposome and niosome preparation using a membrane contactor for scale-up. Colloid. *Colloids Surf., B* **2012**, *94*, 15−21.

(28) Loukas, Y. L. Experimental studies for screening the factors that influence the effectiveness of new multicomponent and protective liposomes. *Anal. Chim. Acta* **1998**, *361*, 241−251.

(29) Shah, S. R.; Parikh, R. H.; Chavda, J. R.; Sheth, N. R. Application of Plackett−Burman screening design for preparing glibenclamide nanoparticles for dissolution enhancement. *Powder Technol.* **2013**, *235*, 405−411.

(30) El-Samaligy, M. S.; Afifi, N. N.; Mahmoud, E. A. Increasing bioavailability of silymarin using a buccal liposomal delivery system: Preparation and experimental design investigation. *Int. J. Pharm.* **2006**, *308*, 140−148.

(31) Shaikh, K. S.; Chellampillai, B.; Pawar, A. P. Studies on nonionic surfactant bilayer vesicles of ciclopirox olamine. *Drug Dev. Ind. Pharm.* **2010**, *36*, 946−53.

(32) Mahmood, S.; Taher, M.; Mandal, U. K. Experimental design and optimization of raloxifene hydrochloride loaded nanotransfersomes for transdermal application. *Int. J. Nanomed.* **2014**, *9*, 4331−4346.
(33) Chaudhary, H.; Kohli, K.; Kumar, V. Nano-transfersomes as a novel carrier for transdermal delivery. *Int. J. Pharm.* **2013**, *454*, 367−380.
(34) Derakhshandeh, K.; Erfan, M.; Dadashzadeh, S. Encapsulation of 9-nitrocamptothecin, a novel anticancer drug, in biodegradable nanoparticles: Factorial design, characterization and release kinetics. *Eur. J. Pharm. Biopharm.* **2007**, *66*, 34−41.
(35) Alund, S. J.; Smistad, G.; Hiorth, M. A. A multivariate analysis investigating different factors important for the interaction between liposomes and pectin. *Colloids Surf., A* **2013**, *420*, 1−9.
(36) Gonzalez-Mira, E.; Egea, M. A.; Garcia, M. L.; Souto, E. B. Design and ocular tolerance of flurbiprofen loaded ultrasound-engineered NLC. *Colloids Surf., B* **2010**, *81*, 412−421.
(37) Pando, D.; Caddeo, C.; Manconi, M.; Fadda, A. M.; Pazos, C. Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol. *J. Pharm. Pharmacol.* **2013**, *65*, 1158−1167.
(38) Pando, D.; Matos, M.; Gutiérrez, G.; Pazos, C. Formulation of resveratrol entrapped niosomes for topical use. *Colloids Surf., B* **2015**, *128*, 398−404.
(39) Devaraj, G. N.; Parakh, S. R.; Devraj, R.; Apte, S. S.; Rao, B. R.; Rambhau, D. Release Studies on Niosomes Containing Fatty Alcohols as Bilayer Stabilizers Instead of Cholesterol. *J. Colloid Interface Sci.* **2002**, *251*, 360−365.
(40) Kremer, J. M. H.; Van der Esker, M. W.; Pathmamanoharan, C.; Wiersema, P. H. Vesicles of variable diameter prepared by a modified injection method. *Biochemistry* **1977**, *16*, 3932−3935.
(41) Pons, M.; Foradada, M.; Estelrich, J. Liposomes obtained by the ethanol injection method. *Int. J. Pharm.* **1993**, *95*, 51−56.
(42) Justo, O. R.; Moraes, A. M. Kanamycin incorporation in lipid vesicles prepared by ethanol injection designed for tuberculosis treatment. *J. Pharm. Pharmacol.* **2005**, *57*, 23−30.
(43) Szoka, F. C., Jr. Preparation of liposome and lipid complex compositions. U.S. Patent 5,549,910, 1996.
(44) Ghanbarzadeh, S.; Arami, S. Enhanced Transdermal Delivery of Diclofenac sodium via conventional liposomes, ethosomes, and transfersomes. *BioMed Res. Int.* **2013**, *2013*, 1−7.
(45) Antonietti, M.; Förster, S. Vesicles and liposomes: a self-assembly principle beyond lipids. *Adv. Mater.* **2003**, *15*, 1323−1333.
(46) Wang, Z.; He, X. Dynamics of vesicle formation from lipid droplets: Mechanism and controllability. *J. Chem. Phys.* **2009**, *130*, 094905.
(47) Lasic, D. D. The mechanism of vesicle formation. *Biochem. J.* **1988**, *256*, 1−11.
(48) Janmey, P. A.; Kinnunen, P. K. J. Biophysical properties of lipids and dynamic membranes. *Trends Cell Biol.* **2006**, *16*, 538−546.
(49) Silva, R.; Ferreira, H.; Little, C.; Cavaco-Paulo, A. Effect of ultrasound parameters for unilamellar liposome preparation. *Ultrason. Sonochem.* **2010**, *17*, 628−632.
(50) Yamaguchi, T.; Nomura, M.; Matsuoka, T.; Koda, S. Effects of frequency and power of ultrasound on the size reduction of liposome. *Chem. Phys. Lipids* **2009**, *160*, 58−62.
(51) Lo, C. T.; Jahn, A.; Locascio, L. E.; Vreeland, W. N. Controlled self-assembly of monodisperse niosomes by microfluidic hydrodynamic focusing. *Langmuir* **2010**, *26*, 8559−8566.

DOI: 10.1021/acs.iecr.6b01552
Ind. Eng. Chem. Res. XXXX, XXX, XXX−XXX