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DEVELOPMENT OF A WATER-IN-OIL-IN-WATER MULTIPLE EMULSION SYSTEM INTEGRATING BIOMIMETIC AQUEOUS-CORE LIPID NANODROPLETS FOR PROTEIN ENTITY STABILIZATION. PART I: EXPERIMENTAL FACTORIAL DESIGN

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ABSTRACT

Lipid nanoballoons integrating multiple emulsions of the type water-in-oil-in-water enclose, at least in theory, a biomimetic aqueous-core suitable for housing hydrophilic biomolecules such as proteins, peptides and bacteriophage particles. The research effort entertained in this paper reports a full statistical 2^3x3^1 factorial design study (three variables at two levels and one variable at three levels) to optimize biomimetic aqueous-core lipid nanoballoons for housing hydrophilic protein entities. The concentrations of protein, lipophilic and hydrophilic emulsifiers, and homogenization speed were set as the four independent variables, whereas the mean particle hydrodynamic size (HS), zeta potential (ZP) and polydispersity index (PI) were set as the dependent variables. The 2^3x3^1 factorial design constructed led to optimization of the higher (+1) and lower (-1) levels, with triplicate testing for the central (0) level, thus producing thirty three experiments and leading to selection of the optimized processing parameters as 0.015% (w/w) protein entity, 0.75% (w/w) lipophilic emulsifier (soybean lecithin) and 0.50% (w/w) hydrophilic emulsifier (poloxamer 188). In the present research effort, statistical optimization and production of protein derivatives encompassing full stabilization of their three-dimensional structure, has been attempted via housing said molecular entities within biomimetic aqueous-core lipid nanoballoons integrating a multiple (W/O/W) emulsion.

KEYWORDS: Aqueous-core lipid nanoballoons, protein entities, water-in-oil-in-water multiple emulsions, structural and functional stabilization, statistical factorial design, optimization.

1. INTRODUCTION

Water-in-oil-in-water (W/O/W) multiple emulsions are examples of versatile colloidal systems in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous (external) aqueous phase.[1-9] The inner aqueous core (where the hydrophilic macromolecular entity is dissolved and/or solubilized) is therefore supported by a lipid matrix surrounded itself by an aqueous surfactant (outer, continuous) phase. Lipid nanoballoons consist of a lipid matrix composed of physiologically compatible lipids[1, 2, 10, 11], and can be stabilized by emulsifiers such as phospholipids and polyoxyethylene ethers.[1-3, 12]

Due to the compartimentalized internal structure of the lipid nanoballoons integrating a W/O/W multiple emulsion, these versatile colloidal systems present clear advantages for encapsulation of hydrophilic macromolecular entities, with a concomitant better control over releasing of such (therapeutic) molecules.[1, 13-17] W/O/W multiple emulsion systems are, however, thermodynamically more unstable and complex than conventional (and simpler) O/W emulsions, mainly due to (i) the higher fluidity of the former (promoted by the lower viscosity of the external aqueous phase), and (ii) the limited weight percentage of lipid used to produce multiple emulsion systems.[3]

When producing a W/O/W multiple emulsion system, the lipid concentration needed to produce the lipid nanoballoons must be kept at low levels, since high lipid concentrations further enhance the thermodynamic instability of these systems and may even promote their rupture (following release of the inner aqueous core under shear rate, with concomitant expulsion of the water-soluble protein entities through the oily layer

between both water phases).[3, 18, 19] To overcome these limitations, two emulsifying agents are required, viz. one with a low value of hydrophilic-lipophilic balance (HLB), added to the lipid phase to stabilize the primary W/O emulsion, and another with a high value of HLB, added to the external aqueous phase to stabilize the secondary (W/O)/W emulsion.[1-3, 7, 13, 18, 20] The two surfactants are needed to stabilize the two existing interfaces in w/o/w multiple emulsions.[1, 2, 13]

Formulating a W/O/W multiple emulsion involves, therefore, optimizing processing parameters such as the composition of the oily phase, both the types and HLB-values of the emulsifiers, the effect of the ratio of hydrophilic/lipophilic emulsifiers, the effect of the oil/water phase ratios, and the effect of processing variables such as homogenization speed, to achieve formation of stable multiple emulsion systems. Hence, a statistical factorial design is mandatory to develop an optimal W/O/W multiple emulsion formulation integrating biomimetic aqueous-core lipid nanoballoons able to house (hydrophilic) protein entities with concomitant structural and functional stabilization of their three-dimensional structure. In the research effort entertained herein, construction of a full statistical 2^3x3 factorial design study aimed at establishing the influence of multiple factors on multiple emulsion properties culminating in the development of a stable W/O/W multiple emulsion system integrating small-sized lipid nanoballoons with aqueous cores, using Softisan100™ as solid lipid, soybean lecithin and poloxamer 188 as low- and high-HLB emulsifiers, respectively, and different high-shear homogenization speeds.

The aqueous core of lipid nanoballoons integrating a W/O/W multiple emulsion system, aimed at mimicking the multifunctional design of biological membranes. The combined effects of protein entity, and lipophilic (soybean lecithin) and hydrophilic (poloxamer 188) emulsifier concentrations in the mean particle (hydrodynamic) size (HS), zeta potential (ZP) and polydispersity index (PI) of the resulting multiple emulsion systems, were thus thoroughly studied via a 2^3x3 factorial design with triplicate testing for the central point, thus allowing not only extraction of a maximum amount of information from a limited number of experiments, but also to establish the influence of multiple factors upon the W/O/W multiple emulsion properties. Since HS and PI are the limiting factors for using the nasal route of administration, the main aim of this experimental full factorial design was to optimize a w/o/w multiple emulsion formulation with appropriate physicochemical parameters for the encapsulation of (hydrophilic) protein entities, envisaging its potential utilization in formulating an isotonic suspension for aerossolization. The resulting (optimized) W/O/W multiple emulsion was subsequently fully characterized physicochemically, and the results produced are the subject of a forthcoming (part II) manuscript.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals. *Lipids*: Softisan 100™ (hydrogenated coco-glycerides consisting exclusively of saturated vegetable fatty acids with chain lengths of $C_{10} - C_{18}$) was a kind gift from Sasol (Sasol Olefins & Surfactants GmbH, Hamburg, Germany). Glycerol (anhydrous) was purchased from Labsynth (Diadema/SP, Brazil).

***Surfactants*:** Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Labsynth (Diadema/SP, Brazil). Kolliphor P188™ (formerly Lutrol F68™, or poloxamer 188) was kindly supplied by BASF ChemTrade GmbH (Ludwigshafen, Germany). Soybean phosphatidylcholine (lecithin) was purchased from Alamar Tecno-Científica Ltda (Diadema/SP, Brazil).

***Other chemicals*:** BSA was purchased from Sigma (St. Louis MO, USA), and was used without further purification. Commercial HCl (37%, w/w) was purchased from ECIBRA – Reagentes Analíticos (Curitiba/PR, Brazil). Tap water was purified in a Milli-Q Elga Purelab system (Molsheim, France) to a final conductivity of ca. 18.2 MΩ.cm^{-1}. The solvents used were all of analytical grade or better, and were used without further purification.

2.1.2. Analytical equipment. The Zeta Potential, Hydrodynamic Size and Polidispersity Index of the multiple emulsion particles were determined in a ZetaPALS system (model NanoBrook 90PlusPALS) from Brookhaven Instruments (Holtsville NY, U.S.A.).

2.2. Experimental procedures

2.2.1. Experimental factorial design. A 2^3x3 full factorial design approach (encompassing three variables each one set at two levels and one variable set at three levels) with triplicate testing for the central (0) level, thus requiring a total of eleven formulations producing thirty three experiments, was applied to fully maximize the experimental efficiency with a minimum of experiments. The three different variables (protein concentration, lecithin concentration and poloxamer 188 concentration) at two levels each, low (-1) and high (+1), and the one variable (stirring speed) at three levels, low (7500 rpm), medium (10000 rpm) and high (12500 rpm), and their influence upon the physicochemical properties of the multiple emulsions produced (MEi) were duly studied. The factorial design undertaken demanded a total of 24 experiments, added with the medium levels for the first three variables which, combined with the three stirring speed levels, produced three additional experiments replicated three times each. The total number of experiments was, therefore, 33. The independent variables were protein concentration, lecithin (lipophilic surfactant) concentration, poloxamer 188 (hydrophilic surfactant) concentration and stirring speed, whereas the established dependent variables under scrutiny were the mean particle (hydrodynamic) size

(HS), Zeta Potential (ZP) and polydispersity index (PI). For each independent variable, the low, medium and high values of the lower, central and upper levels were assigned a (-1), (0) and (+1) sign, respectively (see Table 1). The data gathered was duly analyzed using Minitab® statistical software (release 14.12.0 from Minitab Inc., State College PA, U.S.A.).

Table 1. Full 2^3x3 factorial design, providing the lower (-1), central (0) and upper (+1) level values for each variable.

Independent variables	Levels		
	Low level (-1)	Central level (0)	Upper level (+1)
Protein (%, w/w)	0.005	0.010	0.015
Soybean lecithin (%, w/w)	0.250	0.500	0.750
Poloxamer 188 (%, w/w)	0.500	1.000	1.500
RPM	7500	10000	12500

2.2.2. Production of W/O/W multiple emulsions housing protein entities. The process for producing the water-in-oil-in-water (W/O/W) multiple emulsions housing protein entities was carried out in two high-speed homogenization cycles, using an UltraTurrax (model T25D from IKA Werke GmbH & Co. KG, Staufen, Germany) homogenizer at a constant temperature (ca 39 °C). Two emulsions were prepared sequentially, a primary (simple) emulsion (W_{in}/O), followed by emulsification of this emulsion in another (external) aqueous phase (W_{ext}), thus forming a (second) multiple emulsion of the type water-in-oil-in-water (W_{in}/O/W_{ext}).

2.2.2.1. Preparation of the primary emulsion (W_{in}/O). In a thermostatted bath set at 39 °C, the lipid (Softisan 100™) and the lipophilic emulsifier (soybean lecithin) were melted down in a beaker together with glycerol (constituting the Oily phase) and, in a separate beaker, the internal aqueous phase containing the protein entity was heated up to the same temperature. Following melting of the oily phase, it was added with 1 mL of the internal aqueous phase, followed by high-performance homogenization (10 min at either 7500, 10000 or 12500 rpm). The detailed composition of the Win/O primary emulsion is described next. The inner aqueous phase was constituted by HCl 10 mM, Tween 80 and pure protein entity; the intermediate oily phase encompassed glycerol, Softisan 100™ and soybean phosphatidylcholine; finally, the outer aqueous phase encompassed poloxamer 188 and ultrapure water. Control multiple emulsions were also produced, without protein entity. The aforementioned solid lipid was tested as possible constituent of the oily phase, since it is considered a lipid for modified release formulations. All formulations prepared exhibited a milky and uniform appearance. The oily phase (O) was prepared by melting together 500 mg Softisan 100™ and 250 mg lecithin on a thermostatted bath set at ca. 39 °C and maintained at this temperature. In a separate beaker, 5 mL glycerol was heated up to ca. 39 °C in the same thermostatted bath. For the internal aqueous phase (Win), 5 mL HCl 10 mM and 50 mg Tween 80™ were heated together up to ca. 39 °C in the same thermostatted bath, and added with 25 mg pure protein. When Softisan 100™ and lecithin were melted down, both glycerol and 1 mL of Win were added and thoroughly mixed for 10 min at either 7500, 10000 or 12500 rpm, in the thermostatted bath, thus forming an Win/O emulsion.

2.2.2.2. Preparation of the (W_{in}/O)/W_{ext} multiple emulsion. At the end of the first homogenization cycle, the external aqueous phase (Wext) was then added and a new homogenization cycle performed (10 min at either 7500, 10000 or 12500 rpm). Therefore, final Win/O/Wext dispersions of protein entity were obtained via sequential (optimized) homogenization of a Win/O dispersion involving two cycles at either 7500, 10000 or 12500 rpm for 10 min. The external aqueous phase (Wext) was prepared by dissolving 500 mg poloxamer 188 in 41.4 mL ultrapure water. 20 mL Wext was then heated up in a thermostatted bath set at ca. 39 °C, added to the Win/O emulsion produced earlier, and thoroughly mixed for 10 min at either 7500, 10000 or 12500 rpm. The remainder of the poloxamer 188 solution (maintained at room temperature) was then added to the emulsion thus produced and gently homogenized using a magnetic stirrer at 100 rpm, until room temperature.

2.2.3. Determination of Hydrodynamic Size and Zeta potential. The hydrodynamic size (HS) of the aqueous-core lipid nanoballoons, the polydispersion index (PI) and their Zeta potential (ZP) were obtained in triplicate. To analyze the several multiple emulsions produced by dynamic laser light scattering (DLS), dilutions of samples of the multiple emulsions were prepared following indications of the manufacturer of the analytical equipment (who advocates dilutions in the range of 0.0001% (v/v) - 1.0% (v/v), using an appropriate diluent) (50 µL of multiple emulsion in 20 mL ultrapure water, thus producing a 0.25% (v/v) dilution of the sample, falling well within the dilution range advocated by Brookhaven), duly homogenized using a disposable Pasteur pipette, and analyzed in triplicate in a ZetaPALS (model NanoBrook 90PlusPALS, Brookhaven Instruments, Holtsville NY, E.U.A), thus producing values for the particle mean hydrodynamic size (HS) and polydispersity index (PI), while the Zeta potential (ZP) values were gathered from microelectrophoretic analyses.

3. RESULTS AND DISCUSSION

3.1. Optimization of the multiple emulsion. For producing an optimal W/O/W multiple emulsion formulation, possessing small-sized lipid nanoballoons with aqueous-core, evenly distributed in the emulsion (i.e., with a low polydispersity index), and with a sufficiently low Zeta potential to ensure that no coalescence occurs, the statistical factorial design allowed to produce an emulsion where a larger protein (encapsulated) concentration together with a larger lipophilic surfactant concentration lead to a smaller polydispersion and quite low (negative) Zeta potential values. Centrifugation of the optimized W/O/W multiple emulsion did not lead to any protein liberation induced by bursting nanoballoons (as revealed by UV-VIS spectrophotometry), thus evidencing the firm encapsulation and concomitant structural and functional stabilization of said protein entities, which is in clear agreement with previous findings.[1] Although mechanical stirring energy is important for producing the dispersion, is it not sufficient though; it only overcomes the surface tension barrier during the duration of homogenization. Therefore, the easiest way to stabilize the system is to reduce surface tension, so as to decrease the free energy derived from the expansion of the overall surface area.[21] Thus, in addition to testing several high-speed stirrings, increased surfactant levels were tested in producing the W/O/W multiple emulsions, since tensioactive agents do play an important role in stabilizing emulsions. The emulsifiers should provide an optimum HLB value to stabilize the interfaces. The correct choice of both emulsifiers (lipophilic and hydrophilic) will directly affect formation of the oily droplets. For this purpose, soybean lecithin was the selected lipophilic emulsifier used with a HLB value of 4, and poloxamer 188 (Kolliphor P188™) was selected as hydrophilic emulsifier with a HLB value of 29.[22] However, most surfactants cannot reduce the interfacial tension to levels enough to counteract all the surface free energy caused by the tremendous increase in surface area during homogenization, and thus emulsions (and, particularly, multiple emulsions) are usually considered thermodynamically unstable systems.[1,18,23] The main particularities of nanosized emulsions (with sub-micrometric droplet sizes), making them prime candidates for biopharmaceutical applications, is their greater stability of droplet suspension, a kinetic stability that lasts for months (in clear agreement with the results produced in the present research effort), stability against dilution or even against temperature changes, totally unlike the (thermodynamically unstable) microemulsions *(24)*. Emulsions are generally thermodynamically unstable systems, due to the positive free energy of emulsion formation ($\Delta Gf > 0$). In the mathematical formulation of the second law of thermodynamics, $\Delta G_f = \gamma \times \Delta A - T \times \Delta S_f$ the large positive interfacial energy term ($\lambda \Delta A$) outweighs the entropy of droplet formation (ΔSf), also positive. In the equation just mentioned, λ represents the surface tension, ΔA represents the surface area gained with emulsification, and T represents the temperature. The physical destabilization of emulsions is thus related to the spontaneous trend towards a minimal interfacial area between the two immiscible phases, which can be counteracted by producing suitable values of Zeta potential (either very negative or very positive). And this was indeed achieved in the present research effort, as will be seen in the following sections.

3.2. Hydrodynamic size, polydispersity index and Zeta potential. The results obtained from the analysis by DLS of the several W/O/W multiple emulsions formulated according to the factorial design depicted in Table 1, are displayed in Table 2.

Table 2. Response values (HS, PI and ZP, average (n=3) ± σ) of the three factors depicted in Table 1 for the eleven formulations produced at three different stirring speeds.

Multiple emulsion	Protein (%, w/w)	Lecithin (%, w/w)	P188 (%, w/w)	HS ± σ (nm)			PI ± σ			ZP ± σ (mV)		
				7500 rpm	10000 rpm	12500 rpm	7500 rpm	10000 rpm	12500 rpm	7500 rpm	10000 rpm	12500 rpm
ME01	0.015	0.25	0.50	237.58 ± 2.33	210.47 ± 1.37	140.72 ± 2.83	0.236 ± 0.010	0.257 ± 0.014	0.244 ± 0.021	-32.87 ± 0.86	-30.67 ± 1.72	-28.23 ± 4.18
ME02	0.015	0.25	1.50	234.49 ± 1.20	178.18 ± 6.56	167.39 ± 1.95	0.228 ± 0.022	0.246 ± 0.029	0.256 ± 0.004	-32.59 ± 0.99	-26.34 ± 1.05	-28.25 ± 0.62
ME03	0.015	0.75	0.50	225.77 ± 3.59	195.87 ± 2.36	186.20 ± 2.62	0.259 ± 0.011	0.211 ± 0.012	0.206 ± 0.014	-35.54 ± 1.09	-25.68 ± 2.45	-36.45 ± 0.93
ME04	0.015	0.75	1.50	244.59 ± 5.23	253.49 ± 3.28	219.53 ± 3.35	0.248 ± 0.009	0.241 ± 0.015	0.215 ± 0.022	-38.36 ± 0.62	-26.41 ± 0.64	-32.87 ± 0.51
ME05	0.005	0.75	1.50	279.85 ± 2.66	237.07 ± 22.16	197.92 ± 4.28	0.280 ± 0.007	0.289 ± 0.015	0.236 ± 0.006	-34.20 ±0.05	-28.81 ±2.68	-32.47 ± 1.27
ME06	0.005	0.75	0.50	220.31 ± 2.88	201.13 ± 5.11	212.90 ± 1.39	0.251 ± 0.019	0.241 ± 0.020	0.221 ± 0.013	-34.08 ± 0.53	-32.40 ± 0.74	-34.45 ± 2.14
ME07	0.005	0.25	1.50	244.31 ± 1.55	201.69 ± 0.80	178.71 ± 2.59	0.216 ± 0.026	0.239 ± 0.009	0.262 ± 0.004	-34.39 ± 1.05	-35.07 ± 0.84	-24.17 ± 9.61
ME08	0.005	0.25	0.50	193.92 ± 1.67	163.53 ± 1.54	149.59 ± 2.32	0.218 ± 0.018	0.218 ± 0.014	0.231 ± 0.022	-36.74 ± 1.01	-28.89 ± 3.60	-32.13 ± 1.12
ME09	0.010	0.50	1.00	221.41 ± 2.50	189.11 ± 4.08	184.61 ± 1.46	0.220 ± 0.005	0.248 ± 0.029	0.232 ± 0.012	-31.51 ± 0.71	-31.39 ± 0.90	-35.34 ± 0.37
ME10	0.010	0.50	1.00	219.30 ± 1.55	221.60 ± 0.90	183.29 ± 6.96	0.257 ± 0.013	0.244 ± 0.020	0.242 ± 0.010	-36.14 ± 1.56	-32.79 ± 0.68	-33.68 ± 4.25
ME11	0.010	0.50	1.00	233.54 ± 4.80	179.26 ± 1.99	172.11 ± 2.28	0.245 ± 0.012	0.245 ± 0.009	0.211 ± 0.008	-36.49 ± 0.66	-29.61 ± 1.36	-33.72 ± 1.80

The optimum results evolved from the statistical analysis performed are highlighted in Table 2. The net charge at the surface of particles in suspension affects the ionic distribution in their immediate surroundings, producing an electrical double layer around each particle. When a particle moves, the ions within its boundary move with it, and vice-versa. Zeta potential is the potential that exists at this boundary, with its intensity being an indication of the potential stability in the colloidal system[25], and depends on the concentration of ions in the solvent.

Thus, the main reason why to measure the Zeta potential lies in predicting colloidal stability, which in turn depends on the interactions between particles. The Zeta potential is a measure of the repulsive forces between particles, and since the majority of colloidal aqueous systems is stabilized via electrostatic repulsion, the larger the repulsive forces between particles the smaller the probability for them to become closer and form aggregates, thus leading to a more stable colloidal system. As can be seen in Table 2, the statistical factorial design performed let to production of an optimum multiple emulsion possessing quite homogeneous particles with an average hydrodynamic size of (186.2 ± 2.6) nm and average Zeta potential of (-36.5 ± 0.9) mV, and exhibiting a polydispersity index of 0.206 ± 0.014.

These values were produced for a stirring speed processing parameter of 12500 rpm. For the same (optimized) parameters, a control multiple emulsion was also produced, without encapsulated protein moieties, which produced the following values at time zero: hydrodynamic size of (206.15 ± 8.56) nm and average Zeta potential of (-19.83 ± 2.68) mV, and a polydispersity index of 0.277 ± 0.016.

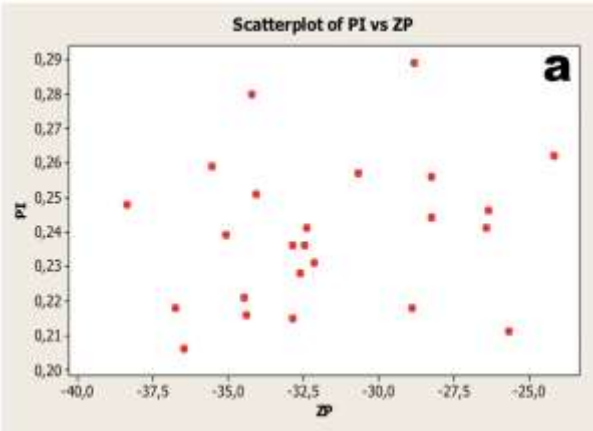
3.3. Statistical analyses. The experimental full factorial design of the type 2x2x2x3 (2^3x3^1) was designed and applied in order to evaluate the influence of four factors in the physicochemical properties of W/O/W multiple emulsions. The factors (independent variables) under scrutiny were (i) protein concentration in two levels, low (-1) and high (+1); soybean lecithin concentration in two levels, low (-1) and high (+1); poloxamer 188 concentration in two levels, low (-1) and high (+1); and homogenization stirring speed in three levels, low (7500 rpm), medium (10000 rpm) and high (12500 rpm). The evaluated dependent variables were the particle average hydrodynamic size (HS), the polydispersity index (PI) and the Zeta potential (ZP).

Combination of the four factors resulted in a total of 24 "treatments" (24 mixtures). Additionally, the central points of the first three factors were also inserted which, combined with the three stirring speed levels, resulted in additional three treatments that were replicated three times each. After each mixture (i.e. W/O/W multiple emulsion) was produced, via combination of the four factors, the variables HS, PI and ZP were measured in triplicate, for each mixture, and the averages (see Table 2) were considered for the statistical analyses of the results.

The statistical analysis performed was the analysis of variance (ANOVA), allowing to evaluate which factors are significant to explain the variation of each dependent variable (HS, PI and ZP).

The significance level adopted for the statistical analyses was 5%, meaning that a factor was considered significant when the descriptive level (*p*-value) for that factor was lower than 0.05. Since HS and PI are the limiting factors for using the nasal route of administration, the main aim of this experimental full factorial design was to optimize a w/o/w multiple emulsion formulation with appropriate physicochemical parameters for the encapsulation of (hydrophilic) protein entities, envisaging its potential utilization in formulating an isotonic suspension for aerossolization.

Response variables HS, PI and ZP did not exhibit any correlation between them (see Figure 1), as can be concluded from the correlation coefficients and *p*-values obtained for PI *vs*. ZP (r = 0.205, *p*-value = 0.338; *p*-value > 0.05, hence no significant correlation exists between PI and ZP), PI *vs*. HS (r = 0.277, *p*-value = 0.191; *p*-value > 0.05, hence no significant correlation exists between PI and HS) and HS *vs*. ZP (r = -0.360, *p*-value = 0.084; *p*-value > 0.05, hence no significant correlation exists between HS and ZP). In this way, these variables could be analyzed individually, i.e., an ANOVA was performed for each one of them according to Montgomery[26], Bates and Watts[27] and Box and Draper.[28] Otherwise, if any correlations were found among these response variables, a multivariate analysis of variance (MANOVA) would be in order.



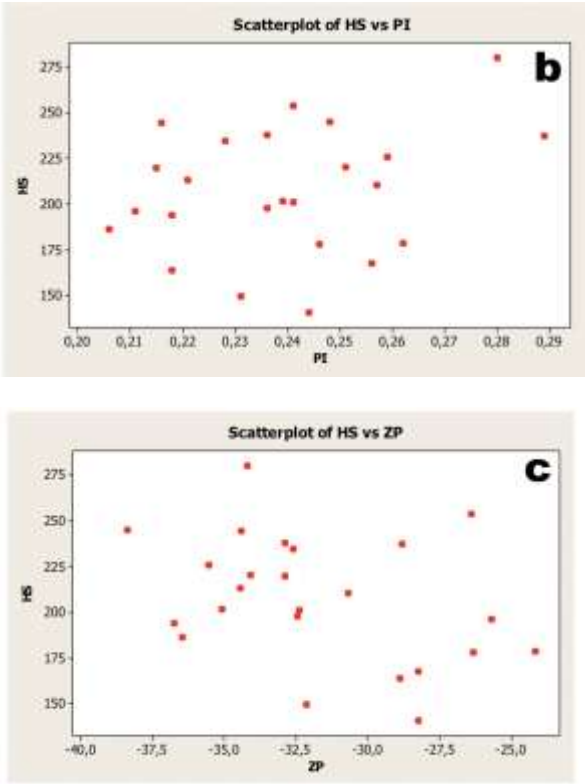


Figure 1: Scatter plots (data means) showing the absence of correlation between PI *vs.* ZP (a), HS *vs.* PI (b), and HS *vs.* ZP (c).

3.3.1. Statistical analysis of the variable HS. The analysis of variance (ANOVA) for the variable HS (particle average hydrodynamic size) (see Table 3) was produced under the assumption that *p*-values lower than 0.05 were statistically significant, i.e., those values whose corresponding effect of the source (factor or interaction) was significant. Thus, from inspection of the data displayed in Table 3 one can see that the factors lecithin concentration (Lecithin), poloxamer 188 concentration (P188) and homogenization stirring speed (RPM) were significant (values in bold in Table 3, under heading HS), meaning that a variation in the levels of these factors leads to a significant difference in the variation of HS. Additionally, and since no significant interaction effects were found, one can conclude that the factors Lecithin, P188 and RPM do promote variations in HS in an independent manner.

Table 3. Statistical analyses of variance (ANOVA) for HS, PI and ZP, using adjusted sum of squares

Source	Degrees of freedom			Sum of squares			Mean square			F-value			*p*-value		
	HS	PI	ZP	HS	PI	ZP	HS	PI	ZP	HS	PI	ZP	HS	PI	ZP
Protein	1	1	1	7.4	0.0001260	7.639	7.4	0.0001260	7.639	0.02	0.95	0.99	0.901	0.355	0.346
Lecithin	1	1	1	5829.7	0.0000920	19.046	5829.7	0.0000920	19.046	12.92	0.70	2.47	**0.006**	0.426	0.151
P188	1	1	1	3730.8	0.0011070	8.402	3730.8	0.0011070	8.402	8.27	8.36	1.09	**0.018**	**0.018**	0.324
RPM	2	2	2	11495.5	0.0003876	128.453	5747.8	0.0001938	64.227	12.74	1.46	8.33	**0.002**	0.282	**0.009**
Protein * Lecithin	1	1	1	154.1	0.0020350	5.358	154.1	0.0020350	5.358	0.34	15.37	0.69	0.573	**0.004**	0.426
Protein * P188	1	1	1	392.9	0.0006100	1.025	392.9	0.0006100	1.025	0.87	4.61	0.13	0.375	0.060	0.724
Protein * RPM	2	2	2	224.1	0.0001116	25.474	112.0	0.0000558	12.737	0.25	0.42	1.65	0.785	0.668	0.245
Lecithin * P188	1	1	1	275.5	0.0002470	0.437	275.5	0.0002470	0.437	0.61	1.87	0.06	0.455	0.205	0.817
Lecithin * RPM	2	2	2	914.0	0.0040716	61.010	457.0	0.0020358	30.505	1.01	15.37	3.96	0.401	**0.001**	0.059
P188 * RPM	2	2	2	165.9	0.0004301	14.519	83.0	0.0002150	7.260	0.18	1.62	0.94	0.835	0.250	0.425
Error	9	9	9	4060.9	0.0011919	69.411	451.2	0.0001324	7.712						
Total	23	23	23	27250.8	0.0104100	340.774									

Note: The values in **bold** and **highlighted in gray** are statistically significant (*p*-value < 0.05)

The levels (-1) for Lecithin, (-1) for P188 and 12500 for RPM were those who promoted the lower values for HS. Additionally, since the interactions between these factors were found not to be significant, one may conclude that the combination of these three levels (-1, -1, 12500) is the one that promotes the lower value for HS. Regarding the protein level, there is no statistically significant difference between the low and high levels.

3.3.2. Statistical analysis of the variable PI. The analysis of variance (ANOVA) for the variable PI (polydispersity index) (see Table 3) shows that there was a statistically significant interaction between the factors Protein and Lecithin, as well as between Lecithin and RPM (homogenization stirring speed); in this way, those factors can not be analyzed independently. The influence of Lecithin on the value of PI depends on the level of Protein as well as on the level of RPM. Additionally, factor P188 was found to be significant, however without interaction with the remaining factors. Hence, factor P188 can be analyzed independently, but the levels of Lecithin must be analyzed within each level of Protein as well as within each level of RPM. The use of Lecithin is essential to decrease interfacial tension between the oily phase and the internal and external aqueous phases, and also to facilitate emulsification of the lipid matrix. Hence, notwithstanding the fact that Lecithin is used due to its high emulsification power able to provide

stabilization of the Wint/O interfaces, it has also been reported to decrease particle size in emulsions, a phenomenon that is mainly explained by its amphiphilic character.[18,29]

In fact, the results obtained in the present research effort for the PI values of the multiple emulsions (which were essentially monodisperse in all cases) were highly dependent on the level of Lecithin used. The PI value is dimensionless and scaled such that, for values smaller than 0.08, the sample is nearly monodisperse; normally, DLS can only give a monomodal distribution within the range 0.05-0.08. For PI values between 0.08 and 0.7, but far apart from 0.7, the sample is essentially monodisperse. Values of PI higher than 0.7 indicate that the sample has a very broad size distribution. The lipophilic portion of lecithin dissolves the lipid phase (i.e. lecithin becomes positioned at the edge of the lipid phase with its lipophilic tails directed towards the lipid phase while the hydrophilic head is directed towards the aqueous phase), hence promoting a long time stabilization in the interface of the emulsions.[18,29] A dependency of PI was found on the concentration of Lecithin, since the complete coverage of the interface is affected by the selected concentration of emulsifier.

The low level (-1) of P188 is the one that promotes the lower value of PI, and Figure 2 shows that for the low level (-1) of Protein it is the low level (-1) of Lecithin that promotes the lower value for PI, whereas for the high level (+1) of Protein it is the high level (+1) of Lecithin the one that promotes the lower value for PI.

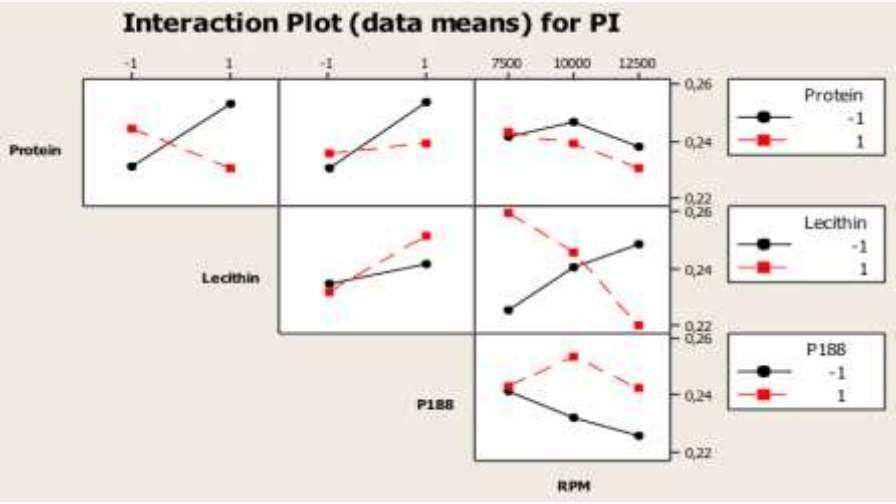


Figure 2: Interaction plots (data means) for PI, showing the contributions of the interactions between Protein, Lecithin, P188 and RPM for the values of PI.

Considering the interaction between RPM and Lecithin one can see that, by fixing the low level (-1) of Lecithin, as the level of homogenization stirring speed increases there is a tendency to obtain higher values of PI (see Figure 2). On the other hand, by fixing the high level (+1) of Lecithin, as the level of homogenization stirring speed increases there is a tendency to obtain lower values of PI (see Figure 2). The variable PI is explained by the model displayed as Equation (1).

Figure 3 displays the three-dimensional surface response plots for the variable PI (polydispersity index), considering all factors that contribute to its minimization.

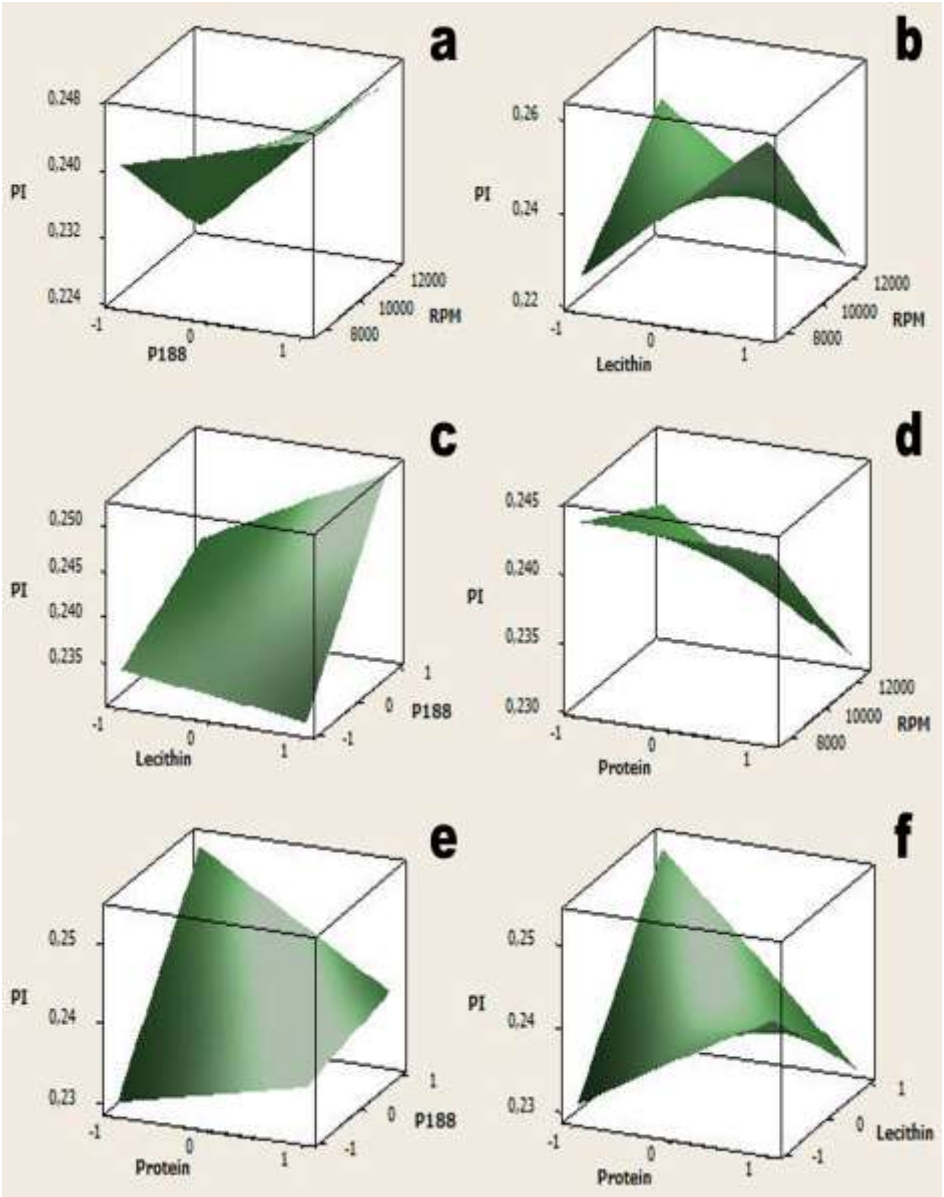


Figure 3: Surface response three-dimensional plots for PI *vs*. RPM and P188 (a), PI *vs*. RPM and Lecithin (b), PI *vs*. P188 and Lecithin (c), PI *vs*. RPM and Protein (d), PI *vs*. P188 and Protein (e), and PI *vs*. Lecithin and Protein (f).

PI = constant + effect of P188 + effect of Protein + effect of Lecithin
+ effect of RPM + effect of the interaction (Protein ∗ Lecithin)
+ effect of the interaction (Lecithin ∗ RPM) + Error (1)

For the interpretation of the interaction between Lecithin and RPM, Figure 2 displays the interactions encountered for the variable PI. From inspection of Figure 2, one can notice that for the lower level of RPM (7500) lecithin in its lowest level (-1) is the one that provides the lower value of PI. However, for the higher level of RPM (12500) lecithin in its highest level (+1) is the one that provides the lower value of PI. Thus, from the statistical analysis performed to the experimental results obtained in the sequence of the full factorial design applied to produce the several W/O/W multiple emulsions, namely to the experimental results obtained for variables HS, ZP and PI of freshly prepared multiple emulsions, it can be concluded that the optimal multiple emulsion (considering a small particle hydrodynamic size associated to the lower polydispersity index) is the one whose formulation parameters in the factorial design are the following ones: Lecithin at the high level (+1), Protein at the high level (+1), Poloxamer 188 at the low level (-1), homogenization stirring speed at the high level (12500 rpm). In the coding performed (see Table 2), the emulsion that corresponds to these parameters has the code ME3/12500.

3.3.3. Statistical analysis of the variable ZP. The analysis of variance performed to variable ZP (see Table 3, under heading ZP) shows that the only significant factor was RPM, whose *p*-value was lower than 0.05. This means that a variation in the levels of RPM promotes a significant variation in Zeta potential (ZP). From inspection of Table 3 it can also be observed that no significant interactions could be found, meaning that the effects of the levels of RPM upon the values of ZP do not depend on the levels of the remaining factors.

4. CONCLUSIONS

In this research effort, development and optimization of lipid nanoballoons housing protein entities integrating a multiple emulsion formulation was pursued, using a lipid with mild melting temperature for the discontinuous oily phase. Departing from the factorial planning designed and utilized in the preparation of the several multiple emulsions, one was able to verify unequivocally how each variable (protein, lecithin and poloxamer 188 concentrations, and homogenization stirring speed) influenced on the physicochemical characteristics of the multiple emulsions. The statistical analysis performed to evaluate the results obtained (average particle hydrodynamic size, average polydispersity index and average Zeta potential) allowed verification of the influence of each parameter in the production of an optimal emulsion. Thus, from the comprehensive statistical analysis performed, it can be concluded that the optimum formulation for encapsulation of protein entities was multiple emulsion 3 (coded ME03) produced with a homogenization stirring speed of 12500 rpm. Two homogenization cycles of 10 min at high level (+1) of stirring speed (12500 rpm), the use of high level (+1) of protein concentration (0.015%, w/w), high level (+1) of lecithin concentration (0.75%, w/w), and low level (-1) of poloxamer 188 concentration (0.50%, w/w), were found to be critical variables for producing stable (aqueous-core) lipid nanoballoon dispersions.

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