CHAPTER 1 HEALTH AREA

NANOSTRUCTURED LIPID CARRIERS FOR CANCER TREATMENT: EFFECT OF PROCESS PARAMETERS ON PARTICLE SIZE AND POLYDISPERSITY INDEX USING EXPERIMENTAL DESIGN

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Abstract

Cancer is a global health problem with high incidence and mortality rates, to address this problem various strategies are being developed. The use of nanosystems for the targeted delivery of anticancer drugs used in chemotherapy is a strategy that has attracted a lot of attention because it promises to improve the efficacy of cancer treatment and reduce side effects, which may have a significant impact on the reduction in cancer mortality.

In the design of anticancer drug delivery nanosystems, aspects such as the size of the particles, the chemistry of their surface, the specificity with which they release the drug at the tumor site and the drug loading capacity, are fundamental to predict the treatment success. Understanding the effect of process parameters that determine the size and stability of drug delivery nanosystems is a major work.

Within drug delivery nanosystems, lipid-based systems have achieved wide success in their clinical application. Lipid particles include micelles, liposomes, solid lipid nanoparticles, and nanostructured lipid carriers. The latter are relevant because they provide greater stability and loading capacity of the drugs than the former.

Therefore, in this work a statistical study was developed to identify the significant variables that affect the size and the polydispersity index, seeking to obtain the conditions to develop nanostructured lipid carriers with small sizes and narrow size distributions. A robust analysis was performed using experimental designs, to provide a basis for the development of these nanosystems with specific sizes (less than 100 nm) with the aim of increase the particle penetration and drug accumulation in the tumor zone for future applications in anticancer drug delivery.

Keywords: cancer, nanostructured lipid carriers, experimental design.

1. Introduction

In the last century, cancer has significantly contributed to the decrease in life expectancy and represents the main cause of death in most countries [1, 2]. Cancer is one of the most important global health problems, in fact, the World Health Organization (WHO) in 2020 indicated that 18.8 million new cases were diagnosed, and 8.97 million deaths associated with this disease were reported [3 – 5]. Cancer, which is the name given to a group of diseases that share similar features, where the main characteristic is abnormal and uncontrolled growth of cells, can occur in almost any type of tissue. There are known more than 100 types of cancers [3]. Breast cancer and lung cancer are the main cause of death in women and men, respectively. A statistical study published in 2021 showed that of the 9.2 million cases of cancer in women (which includes all types) 24.5 % occur in the breast. A worldwide increase in cancer patients is expected in the next 50 years and an incidence of more than 34 million cancer cases is predicted for 2070 [2]. Researchers around the world have been working hard to protect humanity from numerous diseases [6, 7]. Although the advances in medicine have been significant in the last decade and have led to the improvement of existing treatments and the development of new strategies against cancer such as; targeted therapy [8 – 10], chemoradiation [8], vaccine therapies [8], immunotherapy [7, 9, 11, 12], fecal microbiota transplantation [13], archaeal-derived biological nanocarriers [14], infrasound [15], microbiome-associated therapy and host-host relationship [16], RNA (siRNA, miRNA) therapy [17, 18], bacteria-based cancer therapy (BBCT) [19] and cancer treatments based on hyperthermia [20], the administration of free chemotherapeutic drugs is the most widely used therapeutic alternative for the treatment of cancer.

Chemotherapy still shows inherent problems, for example, some drugs have very low solubility due to their bulky polycyclic nature (paclitaxel, etoposide, and docetaxel), which prevents them from hydrogen bonding with water [21, 22]. The poor solubility of drugs limits their bioavailability and reduces the efficacy of cytotoxic treatments. On the other hand, some molecules used as chemotherapeutics are unstable in the gastrointestinal tract and have very low permeability through the intestinal epithelium [22, 23] making them not viable for oral administration. New drugs under development such as 4-(N)-docosahexaenol 2',2'difluorodesoxycytidine show strong antitumor activity *in vitro* and *in vivo* in aggressive cancer models (e.g., pancreatic cancer, breast cancer, lung

cancer, and leukemia), but its clinical application has been limited due to its high instability in the intestine when it is administered in its free form [23, 24].

Similarly, Taxol® (paclitaxel) and Adriamycin® (doxorubicin) are drugs that have required chemical modifications to increase their solubility in water in order to be administered in therapeutic doses [22, 25]. Cancer treatments based on these drugs are not specific and generate side effects [26]. Sustained administration of paclitaxel may cause severe hypersensitivity [27, 28], immunosuppression-related bacterial infections [29], neurotoxicity, haematological cytotoxicity (mainly decreased blood neutrophil count) [30], myalgia [28, 31] and cardiac toxicity. In addition, prolonged use of chemotherapeutic agents can lead to multidrug resistance (MDR), which can greatly compromise treatment success [2, 32].

Alternative strategies for targeting drugs that avoid side effects are necessary. In this area, nanotechnology has been explored for anticancer drug delivery to the tumor site. Nanotechnology is the name given to the sum of those technologies applied in different areas of science and engineering that allow changing the properties and characteristics of materials at molecular and atomic levels [33, 34]. The sizes considered in nanotechnology should be 1–100 nm. These sizes give materials unique properties (optical, electrical, magnetic, etc.) that can be used in fields such as electronics and medicine [35, 36]. In general terms, nanomedicine can be defined as the branch of medicine that makes use of the knowledge and tools of nanotechnology for the prevention, diagnosis, delivery of drugs, repair, and regeneration of biological systems, as well as the monitoring and treatment of diseases through imaging technologies [37 – 40].

Based on their shape, nanomaterials can be classified as 0D (fullerenes, nanowires), 1D (nanotubes, carbon nanofibers), 2D (graphene, nanofilms) and 3D (nanostructured materials, nanocarriers) [41 – 44].

In nanomedicine, chemotherapy drugs are delivered into the body using 3D structures known as nanocarriers. Nanocarriers are used for the encapsulation, transport and targeting of drugs towards the tumor site [38]. Nanocarriers are synthesized from a large number of organic or inorganic precursors, the most popular are: polymeric nanoparticles, lipid nanoparticles (LNPs), hybrid polymer/lipid nanoparticles, carbon nanomaterials, among others [40, 44].

Lipids are amphipathic biomolecules, generally insoluble in water, non-toxic, biocompatible and biodegradable [45 – 47]. Thus, lipid-based nanocarriers have

been widely applied in nanomedicine; particularly, LNPs offer great potential for drug targeting. LNPs include a set of different spherical structures that surround an internal aqueous compartment. In recent years, two groups of LNPs with great therapeutic potential have been developed by combining advantageous properties [47], these are solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) (Figure 1) [48, 49].

SLNs and NLCs are composed of a lipid that is solid at room temperature or a mixture of lipids (solid and liquid) respectively (Figure 1). These nanoparticles generally undergo safe biodegradation [50]. The molecules that make up SLNs and NLCs have minimal influence on the extracellular and intracellular environment due to their chemical and physical similarity to the cell membrane components. These molecules also allow a controlled release of biological compounds [49]. SLNs and NLCs have a low average size (according to the method of synthesis) which allows them to simply flow in the blood avoiding uptake by the reticuloendothelial system (RES). SLNs and NLCs can be modified with various targeting molecules, including peptides, growth factors, aptamers, antibodies, and other small molecules that help them to increase their specificity towards cancer cells [48].

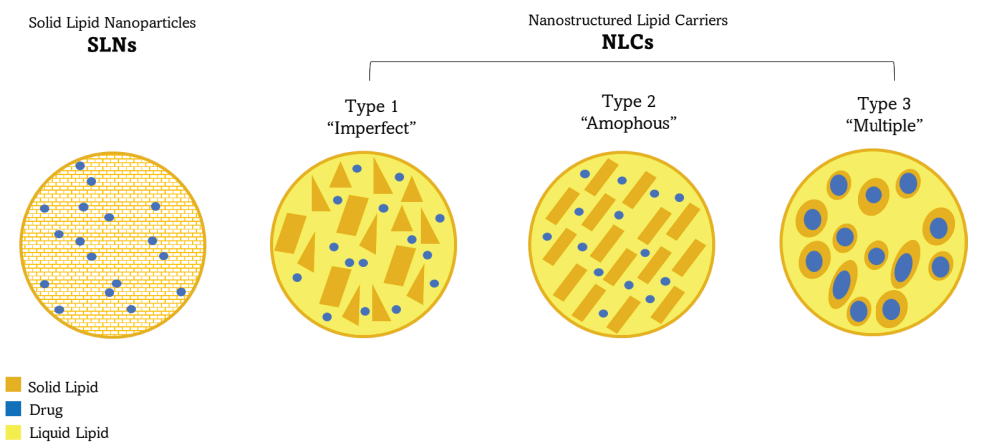

Figure 1. A schematic illustration of Solid Lipid Nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs).

SLNs have the potential to be exploited as drug delivery systems, however, they present a drawback: the crystallinity of the matrix formed within them, caused by the perfect ordering of a single solid lipid, affects the entrapment capacity of the drug, and the chemotherapeutic agent internalized within the matrix

can become expelled from the nanoparticle quickly [49, 50]. As an alternative to SLNs, NLCs were developed; the presence of liquid lipids in the NLCs results in a non-perfect and amorphous network [46 – 51], given the presence of a liquid phase and the disordered structure, there is greater accumulation of the drug in the particle and the encapsulation and load capacity are improved [51].

The lipid mixture, the aqueous phase and the emulsifying agent constitute the main components in the synthesis of NLCs [52]. Low costs, low toxicity and sterilization capacity prior to its medical application are the main properties that materials must have for their use in the manufacture of nanocarriers. In general, the selection of lipids depends on their physiological tolerance, the structure, the solubility of the drug and the miscibility between the mixture of lipids. For the selection of lipids, it must first be considered that these are in the category of molecules generally recognized as safe (GRAS) [53], that is, that they do not produce toxic effects in the concentration employed. In addition, it is imperative to determine the solubility of the drug in the lipid mixture [54]. Triglycerides [55, 56], steroids (cholesterol) [57], waxes [52] and fatty acids [56], among others [58] are lipids commonly used to obtain NLCs.

Surfactants are chemical agents that reduce the surface tension between the lipid phase (organic phase) and the aqueous phase during the production of nanoparticles. These molecules are used as single agents or as mixtures and help to stabilize the lipid dispersion in the aqueous phase. [57, 59]. Some examples of surfactants widely used for lipid nanoparticles formulation include pluronic F68 (poloxamer 188), polysorbates (Tween), polyvinyl alcohol, and sodium deoxycholate (hydrophilic surfactants used in the synthesis of LNPs) [60].

In the last two decades, various techniques have been developed for the synthesis of NLCs, including: high-pressure homogenization (hot and cold) [61, 62], solvent diffusion [63], solvent emulsification-evaporation [64], emulsification sonication [65], microemulsion [66] and solvent injection [67]. The solvent injection method has been useful and more widely used, due to its easy handling and fast production speed, in addition to not requiring sophisticated or robust equipment during the process [52]. Using this technique, it has been possible to obtain particles of 64.00–440 nm [68 – 70]. However, there is not a complex study that analyzes the effect of the factors that influence the synthesis of NLCs to predict the particle size (PS) and obtain different particle sizes with the same composition and synthesis method.

The therapeutic effect of NLCs and nanoparticles in general is closely related to their composition, size, surface charge, and route of administration [21 – 23]. Initially, the design of nanodrugs was based on the enhanced permeability and retention effect (EPR) [71]. The EPR indicates that, in solid tumors, there is a formation of amorphous blood vessels with high permeability of plasmatic components due to the uncontrolled cell growth and the high nutrients demand; this, together with the poor drainage of waste components by the lymphatic system, allows that nanoparticles can easily leak through the capillary openings and reach the tumor stroma; so that, they can accumulate at the tumor site passively [72].

NLCs are highly relevant, and since 2017 more than 200 articles on this subject are published in PubMed annually [73]. The significant increase in the use of these nanoparticles suggests the great potential of NLCs for the treatment of cancer [74 – 79]. Despite the large number of publications, few pharmacological developments based on NLCs are in the final stage of clinical studies for application in humans, in most of them the particle sizes are >100 nm [68 – 70]. For large PS, the passive diffusion process established by the EPR is not the mechanism that promotes the accumulation of particles at the tumor site and other processes such as extravasation and active diffusion (which requires energy expenditure) [80] could be more relevant to enhance the accumulation of nanoparticles at the tumor site. Multiple physiological barriers [22] are involved from the administration of the nanodrug in the bloodstream to its internalization in the cancer cell.

As previously mentioned, the chemical composition and PS are factors that influence the accumulation of nanoparticles at the tumor site. It is still necessary to study the behavior of particles with sizes <100 nm, since most of the investigations focus on PS >100 nm, where the EPR effect has no relevance for the accumulation of NLCs [73 – 79]. To obtain small nanoparticles, which may be useful for the study of accumulation in tumors, the DoE Design of Experiments turns out to be a powerful tool for optimizing the synthesis process evaluating multiple factors [81]. DoE is a structured and organized method to determine the relationships between the factors that affect a process and its output [82]. The use of an experimental design will make it possible to obtain a useful model consciously and accurately for the formulation of particles <100 nm, which can be evaluated in vitro and in vivo with passive accumulation in the tumor site.

2. Materials and methods

2.1. *Materials*

The lipid mixture of the organic phase is composed of 18-carbon phospholipids, stearic acid and oleic acid (Figure 2). Stearic acid (C18, 93661C18H3602 MW:284.48 g/mol, Tm 71 °C, 97 % purity) and oleic acid (C18, 453036/1 12803315, C18H3402, MW:282.47, ρ=0.89 g/mL) were purchased from FlukaTM. Ethanol was used as solvent in the organic phase. Polysorbate 80/ Tween 80 (Hycel 9005-65-6) was used as surfactant. The aqueous phase use PBS phosphate buffer solution as solvent.

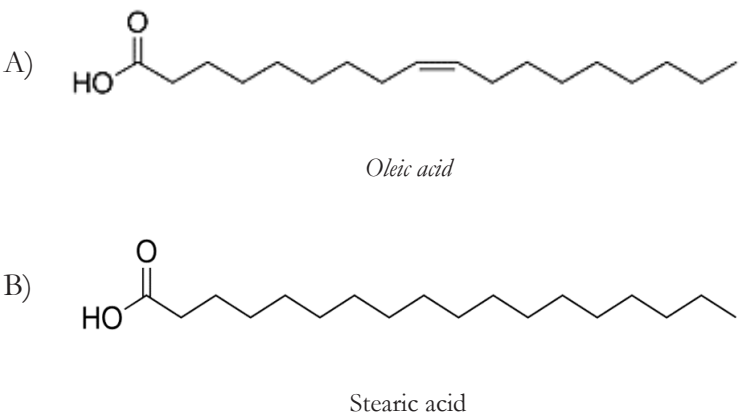

Figure 2. Chemical structure of the lipids used for the synthesis of NLCs. A) Liquid lipid (oleic acid) and B) Solid lipid (stearic acid).

2.2. *Synthesis of NLCs by solvent injection*

To obtain the NLCs, the solvent injection method, reported by Scubert *et al.* was used (Figure 3) [67], with some modifications. This method employs two phases, organic phase (lipid mixture in ethanol) and aqueous phase (surfactant in PBS).

The organic phase was prepared by heating ethanol (solvent) to 70 °C with indirect heat, stearic acid was added to the hot solvent and stirred for 15 min avoiding evaporation. The oleic acid was integrated when the solid lipid was completely dissolved, and it was kept stirring for 30 min. The aqueous phase was prepared by dissolving the necessary amount of surfactant in PBS phosphate

buffer (pH adjusted) at 40 °C and kept warm until synthesis. For the synthesis, the organic phase was rapidly injected into the aqueous phase under high agitation and at high temperature, using a syringe. Subsequently, the nanoparticles were kept stirring (5-15 min). The resulting suspension was sonicated at 70 % power, 45 kHz, for 15 min at 45 °C. The nanoparticle solution was kept at 25 °C for storage.

2.3. *Measurement of particle size and polydispersity index*

Particle size (PS) and polydispersity index (PDI) determination was performed by dynamic light scattering, using a Zetasizer Nano ZS series equipment (Malvern Instruments, USA), after appropriate dilution with PBS. The sample volume was constant (i.e. 1 mL).

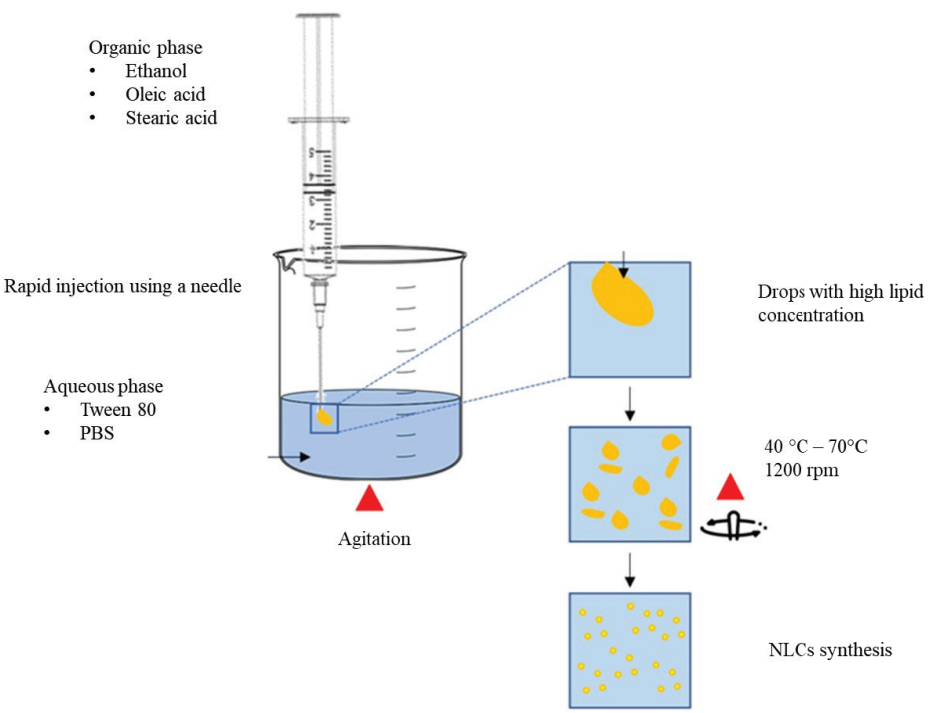

Figure 3. Solvent Injection Method. Obtaining NLCs by injecting a mixture of lipids at high speed in an aqueous phase at high temperature and stirring.

2.4. *Design of Experiments (DoE)*

As previously mentioned, DOE is an appropriate tool for the identification and optimization of critical parameters that interfere in a process [58]. For the selection, evaluation, screening, and optimization of the critical factors during the NLC synthesis, a structured study was carried out as shown in Figura4. First, the design factors were identified using a single factor design. A second step using a screening design allowed irrelevant factors to be discarded during the synthesis process. Subsequently, a full factorial 2^3 design was useful to identify the presence of curvature in the process. Afterwards, a Box-Behnken quadratic model was carried out for the optimization of the process and obtaining a mathematical model for the prediction of the PS and PDI (when the values of the optimized variables were modified). All statistical analysis were performed using Design expert 11 software.

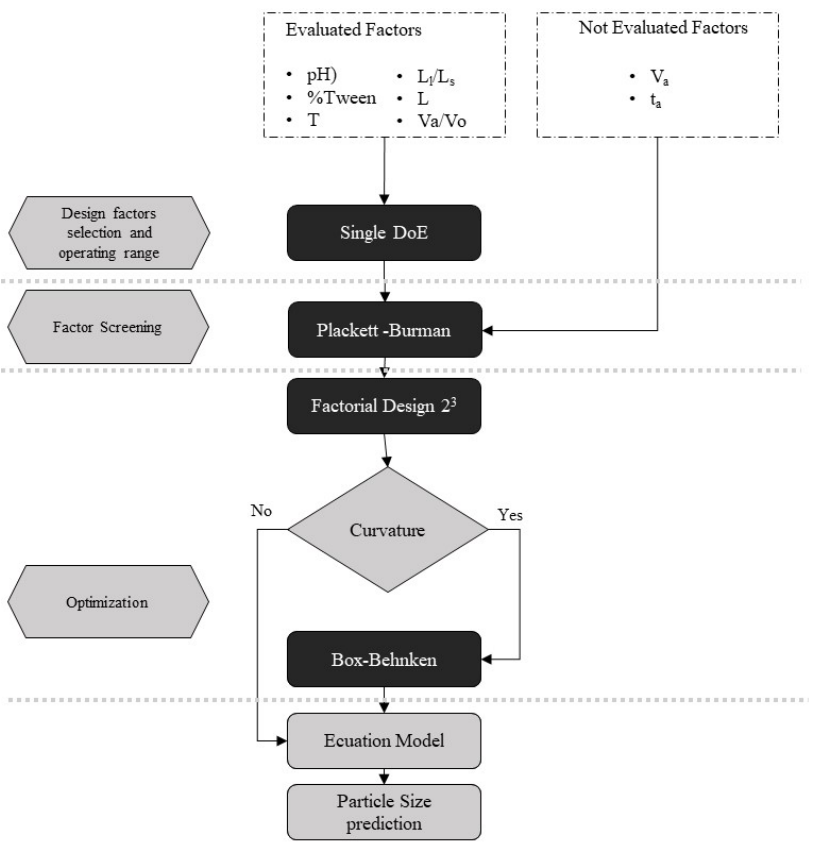

Figure 4. Flow diagram of DoE for the optimization of PS and PDI in obtaining NLCs using the solvent injection method.

2.5. *Selection of factors and operating ranges*

The operating ranges of the design factors were selected based on the effect on PS and PDI, using a single factor experimental design. The variables analyzed were the pH of the aqueous phase (pH), percentage v/v of surfactant (%tween 80), synthesis temperature (T), ratio of liquid lipid/solid lipid in the organic phase (L_l/L_s), total lipid concentration (L) and percentage of the organic phase in the final volume of synthesis (%V_o). The stirring speed (v_a) and stirring time (t_a) during the synthesis were not analyzed in this first phase of the study.

The evaluation of the pH effect was carried out by adjusting the pH of the buffer solution in the range of 3-11 using NaOH and 1.0 M HCl. The synthesis of NLCs was carried out in a range of 30-70 °C for the assessment of the influence of the temperature on the PS and PDI.

Different solutions (Table 1) for %V_o were prepared for the estimation of this factor on the PS and PDI.

Different concentrations of total lipids were evaluated (15, 20, 25, 30 and 35 mg/mL) during the preparation of the nanoparticles. To study the influence of surfactant concentration, different levels of Tween 80 in the aqueous phase were studied: 2 %, 3 %, 4 % and 5 %. It has been observed in previous works that a higher proportion of liquid lipid in the lipid mixture improves the stability of the NLCs [78, 83-86]. To evaluate this factor, the organic phase was prepared with a final concentration

Table 1. Experimental design for the evaluation of the effect of V_o (%) in the synthesis of NLCs.

Organic phase percentage (V_o %)		10 %	20 %	30 %	40 %	50 %
Organic phase						
	Ethanol (mL)	2.50	5.00	7.50	10.00	12.50
	Oleic acid (μL)	22.50	22.50	22.50	22.50	22.50
	Stearic acid (mg)	30.00	30.00	30.00	30.00	30.00
Aqueous phase						
	PBS (mL)	22.50	20.00	17.50	15.00	12.50
	Tween 80 (mL)	1.00	1.00	1.00	1.00	1.00

of total lipids equal to 20 mg/mL, making variations of the proportion of oleic acid from 30 % to 70 %. When one factor was analyzed, the remaining factors were kept constant as indicated below pH=3, %Tween 80=3 %, T=40 °C, L_l/L_s=70 %, L=20 mg/mL, $\%V_o$= 10 %, v_a 1200 rpm and t_a 10 min.

2.6. *Screening of significant variables by Plackett-Burman design of experiments*

Plackett-Burman is a screening design that evaluates and discards irrelevant experimental factors with a minimum of formulations and experimental runs during process optimization [87]. This step is important to eliminate factors that do not significantly affect the response variables. An experimental design of Filtered Plackett-Burman was proposed (Table 2) for the evaluation of the most significant variables during the synthesis of NLCs. Here, the proportion of oleic acid in the lipid mixture (L_l/L_s=70 %) and the percentage of the organic phase ($\%V_o$=10 %) were kept constant in all the experimental runs. Design factors and operating levels are shown in Table 3.

Table 2. Plackett-Burman experimental design runs for screening the significant independent variables affecting PS and PDI during NLCs synthesis.

Run	%tween	L	pH	T	v_a	t	PS	PDI
	(%)	(mg/mL)	-	(°C)	(rpm)	(min)	(nm)	-
1	4	25	3	70	1200	10	18.63	0.185
2	2	25	6	40	1200	10	84.69	0.207
3	4	15	6	70	800	10	11.19	0.158
4	2	25	3	70	1200	5	197.17	0.601
5	2	15	6	40	1200	10	11.01	0.225
6	2	15	3	70	800	10	20.15	0.183
7	4	15	3	40	1200	5	14.35	0.115
8	4	25	3	40	800	10	18.00	0.159
9	4	25	6	40	800	5	14.70	0.266
10	2	25	6	70	800	5	61.82	0.202
11	4	15	6	70	1200	5	12.10	0.163
12	2	15	3	40	800	5	127.17	1.000
13	3	20	4.5	55	1000	7.5	15.45	0.146
14	3	20	4.5	55	1000	7.5	18.18	0.178

Table 3. Level of independent factors selected in Placket-Burman design for screening independent variables.

Factor	Levels	
	(-1)	(1)
% Tween	2	4
L (mg/mL)	15	24
pH	3	6
T (°C)	40	70
v_a (rpm)	800	1200
t (min)	5	10

2.7. *Factorial design*

After discrimination of non-significant variables, the concentration of total lipids (L), concentration of surfactant (% tween 80) and pH of the aqueous solution (pH) were selected for a 2^3 factorial design. The effects of the factors were examined at two levels (+1 and -1) as shown in Table 4. The values of the levels were selected based on the results of the previous analysis (Plackett-Burman). For the experimental design process, nine different formulations were prepared and carried out in triplicate (27 runs) (Table 5). Statistical analysis was performed with Design Expert 11 software. For this design, the following factors were kept constant as indicated: pH=6, T=70 °C, L_l/L_s=70 %, $\%V_o$=10 % and v_a=1200 rpm.

2.8. *Box Benhken quadratic design*

After the system was characterized and the important factors were identified in a reasonable and accurate way (Table 6), the next objective was optimization. Using an optimization model, also called Response Surface/Box-Benhken (Table 7), levels +1, 0 and -1 were evaluated to obtain response surface plots and the mathematical model that describes the effect of the significant factors in the response variables PS and PDI, related to the process of obtaining the NLCs. The software was used to determine combinations of the factors studied to obtain NLCs of different sizes.

Table 4. Level of independent factors selected by screening method for full factorial 2^3 design.

Factor	Levels	
	-1	1
% Tween	2	4
L (mg/mL)	15	24
t (min)	5	10

Table 5. full factorial design 2^3 for robustness study.

Run	% Tween 80	t (min)	L (mg/mL)	PS (nm)	PDI
1	2	5	15	117.6	0.333
2	2	5	15	110.7	0.529
3	2	5	15	108.9	0.501
4	4	5	15	16.08	0.154
5	4	5	15	16.18	0.17
6	4	5	15	16.05	0.146
7	2	10	15	107.3	0.459
8	2	10	15	112	0.313
9	2	10	15	100.7	0.313
10	4	10	15	18.41	0.238
11	4	10	15	18.25	0.236
12	4	10	15	16.29	0.144
13	2	5	25	108.8	0.274
14	2	5	25	110	0.349
15	2	5	25	114.5	0.297
16	4	5	25	21.26	0.241
17	4	5	25	19.26	0.172
18	4	5	25	19.7	0.18
19	2	10	25	125.8	0.261
20	2	10	25	131.4	0.283
21	2	10	25	131.5	0.334
22	4	10	25	19.79	0.193
23	4	10	25	19.47	0.192
24	4	10	25	19.3	0.192
25	3	7.5	20	32.44	0.294
26	3	7.5	20	32.33	0.302
27	3	7.5	20	32.16	0.301

Table 6. Factor levels for Box Benhken response surface methodology.

Factor	Levels	
	-1	1
tween %	1.5	4.5
L (mg/mL)	15	30
t (min)	5	15

Table 7. Experimental design matrix for Box-Behnken Response Surface methodology.

Run	Tween 80 (%)	t (min)	L (mg/mL)	PS (nm)	PDI
1	1.50	5	22.5	104.20	0.420
2	4.50	5	22.5	15.39	0.186
3	1.50	15	22.5	92.00	0.300
4	4.50	15	22.5	14.66	0.166
5	1.50	10	15.0	29.61	0.510
6	4.50	10	15.0	13.75	0.170
7	1.50	10	30.0	116.00	0.406
8	4.50	10	30.0	21.36	0.307
9	3.00	5	15.0	70.23	0.122
10	3.00	15	15.0	15.34	0.225
11	3.00	5	30.0	95.25	0.693
12	3.00	15	30.0	132.00	1.000
13	3.00	10	22.5	18.41	0.164
14	3.00	10	22.5	17.66	0.240
15	3.00	10	22.5	19.32	0.249

3. Results and Discussion

3.1. *Effect of independent factors on the synthesis of NLCs*

3.1.1. Effect of the pH of the aqueous solution on PS and PDI during the synthesis of NLCs

As shown in Table 8, the PS of the NLCs obtained varies from 22.8–3511 nm with a PDI of 0.243 to 1.000 when NLCs were synthesized at pH 3-11. When alkaline solutions (pH>pKa) were used, the PS underwent a significant increase, related to the ionization state of the fatty acids in the synthesis medium. The

lipids used in the mixture have an acidic character with pKa values of 10.15 and 9.85 for stearic acid and oleic acid, respectively [88]. When the lipids are in a medium with a pH greater than their pKa, the molecules reduce the ionization state and therefore the repulsion between them, thus causing a crystallization process that results in the aggregation of the molecules and therefore in the increase in PS [89]. Likewise, under alkaline conditions there is no uniformity in the particle size (PDI 0.900-1.000) and a polydisperse solution is obtained. On the other hand, acidic conditions of the aqueous solution (pH<pKa) produced a better particle size distribution (PDI 0.243-0.270) and clearly the size of NLCs decreased (PS 22.68-31.37 nm) (Figure 5), which results convenient when we want to increase stability and storage time, since it has been reported that larger particles have less stability during storage time [55]. The pH of the aqueous phase is also relevant when it is desired to integrate a drug into the nanocarriers, the pH conditions will also influence the ionization of the drug and could increase or decrease the solubility, which will be reflected in the efficiency of drug entrapment and release [73, 90].

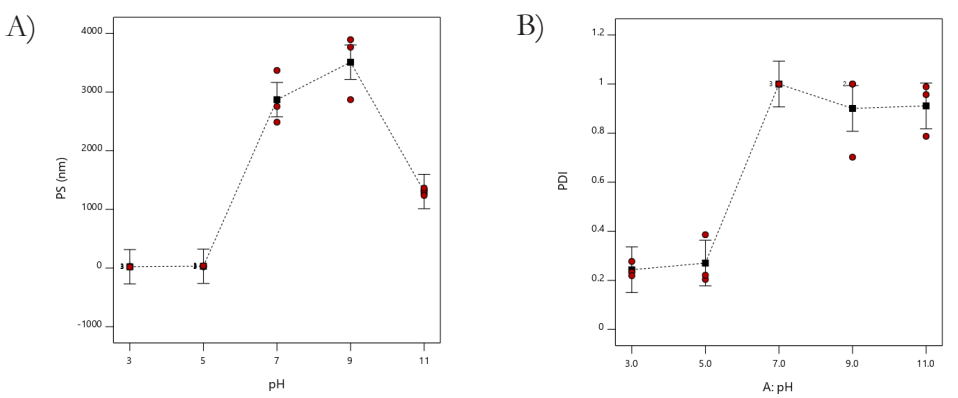

Figure 5. Effect of the aqueous solution pH on
A) PS and B) PDI in NLCs synthesis. $p<0.05$, $R^2=0.97$.

3.1.2. Effect of temperature on PS and PDI during the synthesis of NLCs

The effect of temperature on PS during NLC synthesis is shown in Table 8 and Figure 6. After statistical analysis, no significant effects were observed in the different treatments ($p<0.05$) and PS was in the range of 15.04-19.93 nm. The highest PS was observed when the synthesis temperature was 30 °C and although lower synthesis temperatures were not analyzed, it has been observed in previous

Table 8. Evaluated factors by single-factor design during the NLCs synthesis.

Factor		PS (nm)	PDI
pH			
	3	22.68 ± 6.00	0.243 ± 0.030
	5	31.37 ± 8.82	0.270 ± 0.100
	7	2871 ± 452	1.000 ± 0.000
	9	3511 ± 557	0.900 ± 0.172
	11	1305 ± 63.4	0.921 ± 0.116
Temperature (°C)			
	30	19.93 ± 7.40	0.160 ± 0.026
	40	16.17 ± 0.11	0.155 ± 0.005
	50	15.61 ± 0.38	0.146 ± 0.021
	60	15.99 ± 0.54	0.189 ± 0.008
	70	15.04 ± 0.44	0.149 ± 0.003
% V_o			
	10	16.67 ± 0.98	0.204 ± 0.035
	20	21.55 ± 1.36	0.231 ± 0.086
	30	821 ± 651	0.621 ± 0.344
	40	4567 ± 1146	1.000 ± 0.000
	50	2867 ± 1218	1.000 ± 0.000
L (mg/mL)			
	15	15.3 ± 1.32	0.136 ± 0.050
	20	17.2 ± 0.08	0.199 ± 0.080
	25	17.85 ± 0.96	0.283 ± 0.060
	30	102.69 ± 14.78	0.383 ± 0.081
	35	125.30 ± 36.60	0.430 ± 0.123
% tween 80			
	2	60.43 ± 5.67	0.208 ± 0.009
	3	30.27 ± 5.46	0.389 ± 0.004
	4	17.22 ± 0.26	0.510 ± 0.019
	5	16.44 ± 0.36	0.218 ± 0.003
% L_l/L_s			
	30	18.13 ± 0.90	0.194 ± 0.007
	40	17.98 ± 2.43	0.156 ± 0.030
	50	20.45 ± 3.18	0.198 ± 0.084
	60	19.91 ± 2.69	0.256 ± 0.020
	70	17.78 ± 0.96	0.203 ± 0.019

works [56] that PS increases when working at 20 °C. It is convenient to work at temperatures higher than the melting point of the solid lipid used in the organic phase mixture (stearic acid Tm=71 °C) [91], because, although it is not reflected in the PS, the structure and morphology of the NLCs is affected and may not be uniform. When working at low temperatures, the fast solidification and formation of the lipid network during the formation of NLCs (when the organic phase is rapidly injected into the aqueous phase) can cause low encapsulation of the drug [65, 75] and produce particles of variable composition that will give less stability to the particle suspension. Due to this, and because there are no differences between experimental treatments, the working temperature was kept constant at 70 °C during the subsequent optimization phases.

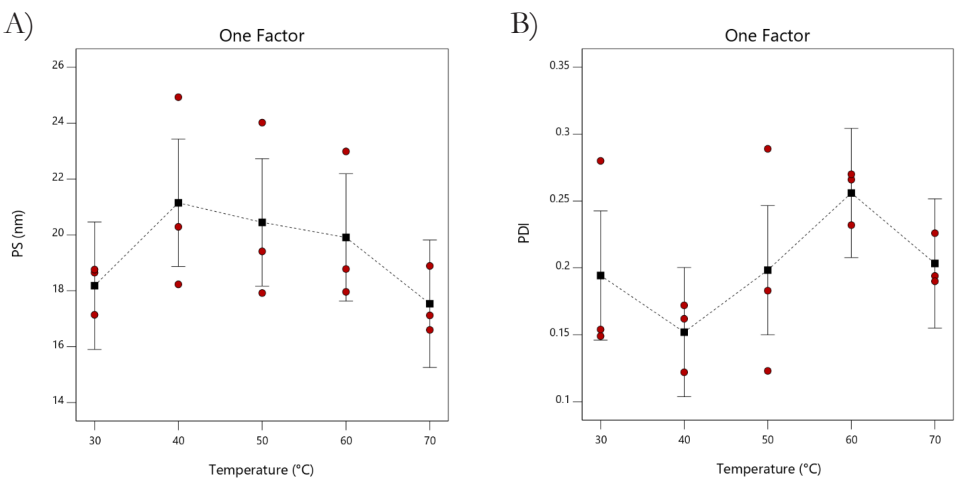

Figure 6. Effect of the temperature on A) PS and B) PDI in NLCs synthesis. $p<0.05$, $R^2=0.31$.

3.1.3. Effect of $\%V_o$ on PS and PDI during the synthesis of NLCs

Different volumes of ethanol during the synthesis of NLCs were used to study the effect of $\%V_o$. As shown in Table 8 and Figure 7, the increase in $\%V_o$ results in a considerable increase in PS, but even more so in PDI, which drastically changes from 0.204 to 0.621 when the ethanol volume is increased from 10 % to 30 %. The stability of NLCs (data not shown) is considerably affected and percentages of 20 % ethanol led to the separation of the phases (organic and aqueous) in less than 24 h. As suggested by Scubert *et al.* [67], it is crucial to avoid exceeding the critical solvent/water ratio as this would result in coarser particles with large PS.

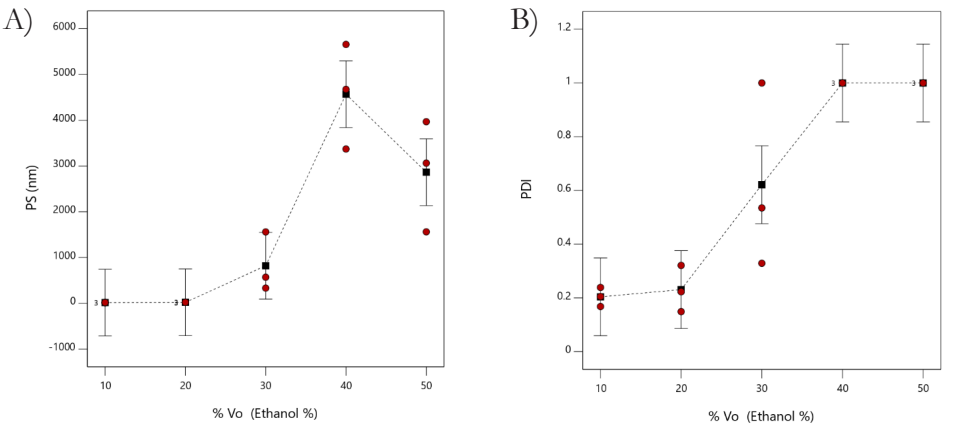

Figure 7. Effect of $\%V_o$ on A) PS and B) PDI in NLCs synthesis. *p>0.05, R^2=0.88.*

3.1.4. Effect of total lipid concentration (L) on PS and PDI during the synthesis of NLCs

Consistent with previous research [77, 78, 85], where the PS was considerably affected in direct proportion to the amount of total lipids dissolved in the organic phase, in this work PS increased from 15.3 to 125.30 nm (Figure 8) when lipid concentration increased. Harshad *et al.* evaluated different levels of lipid concentration achieving a decrease from 349.2 nm to 218.6 nm when working from a high level to a low level of lipid concentration [77]. In this part of the process (evaluation of independent factors) the lipid concentration varied from 15-35 mg/mL. No significant effects were observed at first treatments of 15 to 25 mg/mL, however, increases beyond 30 mg/mL led to larger PS. These results can be attributed to the increase in the viscosity of the organic phase, which makes it difficult to break the lipid droplets formed when they are injected into the aqueous phase. In the same way, these results suggest that the lipid concentration, as one of the factors with greater ease of control, could be a critical factor for the optimization of the process. Since the objective of the study is to obtain particle sizes ≤100 nm, the operating range of this variable for subsequent analyzes was established at 15-25 mg/mL.

3.1.5. Effect of surfactant concentration in the aqueous phase on PS and PDI during the synthesis of NLCs

Those NLCs prepared with the lowest concentration of surfactant (2 % Tween 80) showed a considerably large PS (60.40 nm) compared to the rest of the treatments (Figure 9). The gradual addition of Tween 80 results in smaller

particle sizes [92]. The sizes of NLCs obtained are the result of the reduction of the surface tension between the organic and aqueous phases, which inhibits the aggregation of small droplets during lipid injection [93]. Increases of 4 % to 5 % surfactant in the aqueous phase do not further reduce the particle size, however, smaller sizes are not required in the process. For all the above, 4 %-2 % Tween 80 was selected as the operating range for the screening designs.

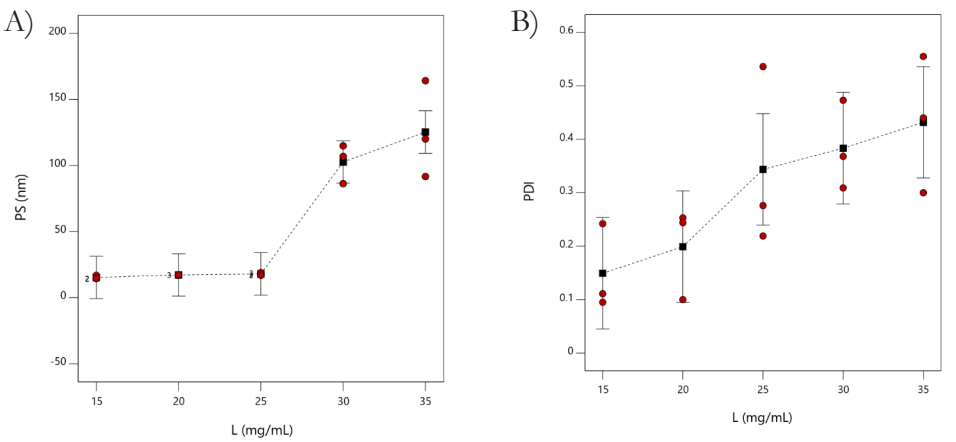

Figure 8. Effect of lipid concentration in the organic phase (L) on A) (PS) and B) (PDI) of NLCs. $p<0.05$, $R^2=0.92$.

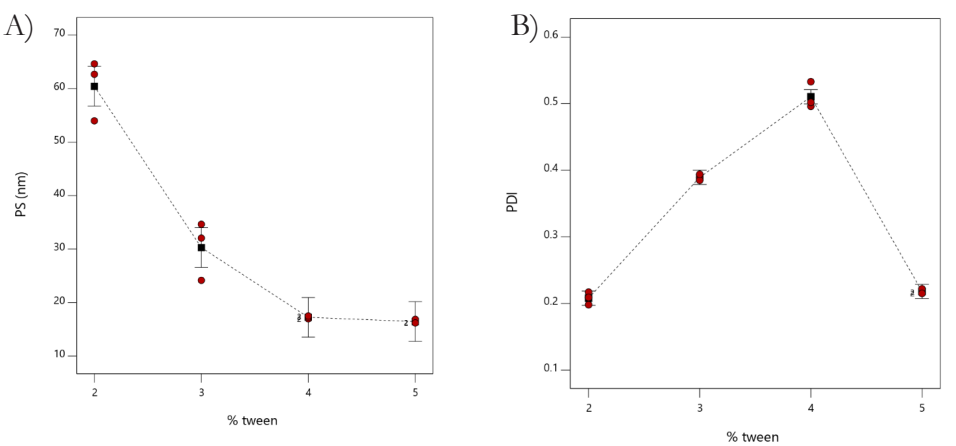

Figure 9. Effect of the surfactant concentration (%tween 80) on A) (PS) and B) (PDI) of NLCs. $p<0.05$, $R^2=0.99$.

3.1.6. Effect of the percentage of liquid lipid in the mixture on the PS and PDI during the synthesis of NLCs

In order to investigate the effect of the proportion of oleic acid in the lipid mixture, 5 different oleic acid:stearic acid (30-70 %) formulations were prepared (Table 8). Figure 10 shows that there are no significant changes in both PS and PDI response variables between the treatments. The PS were from 17.98 to 20.45 with a PDI 0.156-0.256, indicating that the particles exist in a monodisperse solution at all levels.

Previously, Chahinez *et al.*, evaluated liquid and solid lipid variations in different mixtures (triglycerides, short, medium and long chain phospholipids, glycerols, etc.) [79], showing multiple effects in all of them, however, the results of the study in medium chain phospholipids (MCF) are comparable with those obtained in this study (which uses two MCF), since there are no significant changes in PS when the percentage of liquid lipid increases. Since there are no significant differences in the response variables and since the high percentages of liquid lipid in the mixture increase the stability of the nanocarriers, it was decided to work with 70 % oleic acid in the subsequent experimental designs.

3.2. *Plackett-Burman (PB) screening design*

Plackett-Burman (PB) designs are filtering designs that involve a large number of factors and relatively few experiments [87]. PB has been widely used for the identification of the most significant independent factors affecting a process. A total of 14 experiments were performed involving six independent factors as shown in Table 2. The independent factors and their levels are shown in Table 3. The selected response variables (PS and PDI) exhibit great variation suggesting that the independent variables have a significant effect on them. The analysis of variance (ANOVA, Table 9, Table 10) for both response variables confirms that only some factors are relevant in the synthesis of NLCs. T and v_a ($p>0.05$) do not have significant effects during the synthesis of NLCs.

On the other hand, the surfactant concentration and the agitation time ($p<0.05$) are really significant, and it is suggested that they are two of the factors that govern the PS, which was verified in subsequent analyses. The statistics for L and pH show different values in both ANOVAs and suggests that they may not influence the PS. The screening analyzes are not used for the optimization and obtaining of a mathematical model [87], but rather as a method of selecting

variables for more robust methods, since in PB the effects of some factors may be hidden by the alias formed between them [94]. For this reason, although L is not significant in this part of the study, it was decided to include it in the factorial designs, since previous tests (Section 3.1.4) [67, 76, 77, 85] have shown a significant effect on PS, when there are variations in lipid concentration.

Table 9. DoE Plackett-Burman ANOVA for PS.

Source	SS	SM	F-value	p-value	
Model	35284.07	5040.58	7.47	0.0209	*significant*
%tween	14129.54	14129.54	20.93	0.006	
L	3343.67	3343.67	4.95	0.0766	
pH	3289.81	3289.81	4.87	0.0784	
T	224.21	224.21	0.3321	0.5894	
v_a	611.33	611.33	0.9055	0.385	
t	5760.14	5760.14	8.53	0.033	

Table 10. DoE Plackett-Burman ANOVA for PDI.

Fuente de variación	SS	SM	F-value	p-value	
Model	0.6995	0.0874	6.6	0.0263	*significant*
%tween	0.1402	0.1402	10.58	0.0226	
L	0.0071	0.0071	0.5325	0.4983	
pH	0.0988	0.0988	7.46	0.0412	
T	0.0142	0.0142	1.07	0.3478	
v_a	0.0249	0.0249	1.88	0.2285	
t	0.1419	0.1419	10.71	0.0221	

3.3. *Factorial design 2^3*

During the preliminary studies, three significant design variables were determined: % tween 80, L and v_a . A factorial design allows detecting possible interactions between these factors [95], which may affect the NLCs synthesis process. The factorial design is a much more effective tool to interpret and implement the results of the study of the process, considering simultaneous changes in the parameters studied. The effects of % tween, L and v_a were evaluated on the response variables PS and PDI using a 2^3 factorial design (Table 4, Table 5).

24 experimental runs (8 tests in triplicate and 3 central points) were prepared and the NLCs were synthesized by the solvent injection method.

As mentioned in previous works, the presence of a surfactant is necessary and irreplaceable for the formulation of NLCs [55, 73, 90], but there is a limit that can be used to avoid being irritant and toxic. For this reason, the objective of evaluating its interaction with other process variables is to minimize the concentration of tween 80. The results obtained were treated statistically by ANOVA and it was determined that the design model is significant ($p<0.05$) and is capable of describe more than 99 % of the events that occurred for the PS (Table 11). Clearly the %tween 80 factor is the most significant during the synthesis of NLCs. Center points were used in this design, since factorial designs assume that there is a linear relationship between each X & Y. Therefore, if the relationship between any X and Y shows curvature, a factorial design should not be used because the results may not be reliable [96]. Then, the ANOVA (Table 11) concludes that curvature exists, and it is necessary to use a response surface experimental design (RSM). Although this design (factorial 2^3) can detect curvature and predict some responses, an RSM must be used to model the curvature and acquire a fitted mathematical model.

Table 11. ANOVA of factorial design 2^3.

Source	Sum of quares	Mean Square	F-value	p-value	
Model	56925.88	8132.27	968.5	< 0.0001	significant
Tween %	55985.5	55985.5	6667.48	< 0.0001	
t	70.66	70.66	8.41	0.0095	
L	282.36	282.36	33.63	< 0.0001	
(tween)* % (t)	51.69	51.69	6.16	0.0232	
(tween)* % (L)	93.14	93.14	11.09	0.0037	
(t)*(L)	183.15	183.15	21.81	0.0002	
(tween)* % (t)*(L)	259.38	259.38	30.89	< 0.0001	
Curvature	3141.88	3141.88	374.18	< 0.0001	

3.4. *Box-Behnken /Response Surface Method*

The response surface method allows to evaluate a limited number of variables at different levels with a small series of experiments [85]. This approach was used selecting the experimental level for each variable based on the results of preliminary experiments. The surface and contour plots (Figure 10) show the interaction

of different factors on PS. The influence of the factors investigated on the PS using Box Behnken is shown in Table 7.

The ANOVA statistical analysis (Table 12), when the model was adjusted eliminating non-significant interactions, confirms that the model is significant ($p<0.05$) and that it can describe 97 % of the events. Table 7 shows that the PS can vary from 13.75-132 nm, suggesting it to be an adequate model for obtaining small particle sizes of NLCs useful in nanomedicine applications against cancer.

Analyzing the coded equation (Eq. 1), the most significant factor contributing to the variation in PS was the concentration of tween 80, this is evident when observing the value of its coefficient. The %tween 80 factor shows a negative effect on the PS, which translates into a decrease in the size value, thus being favorable for obtaining smaller particles, necessary for this study, and a monodisperse solution of particles. The observation of the increase of the PS with the increase of the concentration of lipids (L) in the organic phase had already been observed in this study and in previous works [83 – 86]. This can be associated with the observations presented in section 3.3.4, where the increase in the viscosity of the medium, caused by the increase in the percentage of lipids, and the difficulty in breaking the lipid droplets, is reflected in the particle size.

$$PS(nm) = 20.02 - 34.58\, Z_1 + 29.46\, Z_3 - 19.70\, Z_1 Z_3 + 22.91 Z_2 Z_3 + 35.37\, Z_2^2 + 23.98 Z_3^2$$

Eq. 1

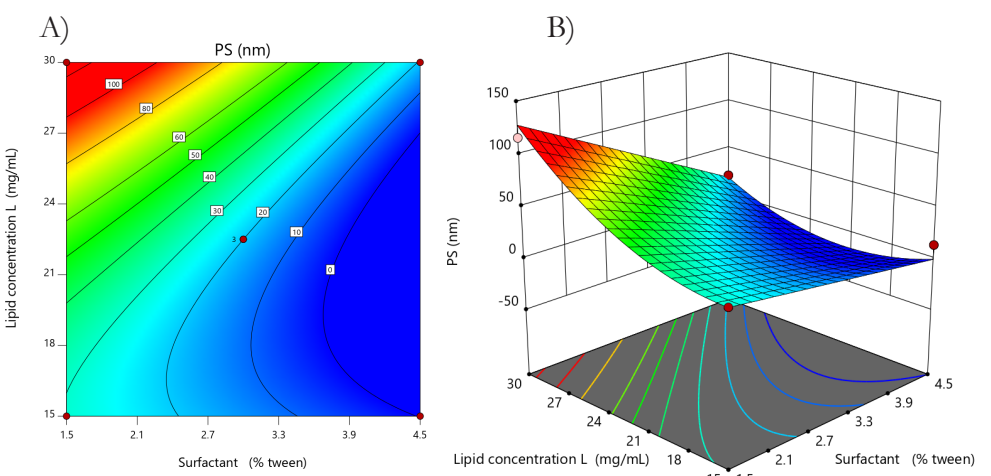

Figure 10. Influence of investigated parameters on PS: (A) counter plot and (b) surface plot $p<0.0001$, $R^2=0.97$.

Table 12. ANOVA of factorial design 2^3.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	26526.01	6	4421	41.44	< 0.0001	significant
%tween	9566.9	1	9566.9	89.68	< 0.0001	
L	6943.13	1	6943.13	65.09	< 0.0001	
(%tween) (L)	1551.57	1	1551.57	14.55	0.0051	
(v_a) (L)	2099.47	1	2099.47	19.68	0.0022	
v_a^2	4645.9	1	4645.9	43.55	0.0002	
L^2	2136.65	1	2136.65	20.03	0.0021	

3.5. *Optimization*

The Design Expert 11 software was used as a tool to determine the values of the different process factors, when a certain particle size is established. The values for each factor when it is desired to obtain particles with PS of 20, 60 and 100 nm are shown in Table 13. The experimental results show that the model is useful for predicting PS and PDI.

Table 13. Predicted and Experimental PS using RSM.

Tween (%)	v_a (min)	L (mg/mL)	Predicted PS (nm)	Experimental PS (nm)	Predicted PDI	Experimental PDI
4.37	14.51	23.13	20	19.25 ± 0.45	0.265	0.253 ± 0.02
2.57	5.54	17.01	60	71.35 ± 4.50	0.246	0.136 ± 0.13
2.74	12.26	27.73	100	102.93 ± 2.19	0.481	0.426 ± 0.01

When nanocarriers are used, the particle size is a determining factor in increasing the efficacy of cancer treatments. Previous work has shown the importance of particle size and distribution, for example, Caster *et al.*, in 2017 [96] demonstrated, by comparing 50, 100 and 150 nm particles in in vitro studies, that particles with a size of 50 nm and a better size distribution between them can more easily penetrate cells and carry out their therapeutic effect. A small particle size allows to increase the circulation time in the blood, by being able to evade RES. If a smaller particle size is enough to evade the immune system, the use of polyethylene glycol (PEG) can be limited. Recently, a particle size less than 100 nm is

a frequently observed feature in cancer treatment and most medically approved nanodrugs are usually >50 nm in size [91].

To identify in subsequent work whether particle sizes smaller than 100 nm are sufficient to evade all biological barriers, it is relevant to obtain small-sized nanocarriers, with a narrow distribution, but all of them with the same chemical composition.

4. Conclusions

In this study, NLCs were obtained by solvent injection method. Despite the simplicity of the technique, the solvent injection method has not been extensively studied to analyze the factors involved in NLC synthesis. Previous works had been analyzing the effect of independent variables (a single factor at a time) on the PS and the PDI, thus ignoring the interactions between independent factors. The DoE is a useful method to discriminate irrelevant factors in the production process of NLCs and based on a series of precise and well-founded experimental designs, it manages to determine the factors that have a significant effect on the synthesis of nanocarriers.

Using DoE and the solvent injection method, eight process factors (pH, %tween, T, L_l/L_s, L, %Vo, v_a and ta) that directly affect the PS and PDI of the NLCs were evaluated. By evaluating each factor independently, it was determined that the percentage of solvent and the percentage of liquid lipid in the lipid mixture do not have a real effect on PS and PDI and work levels other than the established critical values ($\%V_o$ =10 % and L_l/L_s, =70 %) destabilizes the particle suspension. Using Plackett-Burman, the temperature and the stirring speed were discriminated, since they do not present significant effects during the process. For the factorial experimental design, only %tween, L and v_a were used, the presence of curvature suggested adjusting the design to a quadratic model using RSM/Box-Benhken. The quadratic model indicates that two factors are critical during the synthesis of NLCs; firstly, the surfactant concentration negatively affects the particle size, allowing small particle sizes, which is convenient to obtain particles with PS<100 nm. On the other hand, the concentration of total lipids is another critical factor that will directly affect the size of the nanocarriers when their levels increase, that is, a higher concentration of lipids in the aqueous phase promotes particles with PS>100 nm.

The adjusted method is useful to predict the PS when variations of %tween and L (maintaining constant pH=6.0, T=70 °C, L_l/L_s L=70 %, $\%V_o$=10 %, v_a=1200 rpm and t_a=10 min) are performed. With the model adjusted it is

possible to obtain NLCs with PS 20, 60 and 100 nm with the same chemical composition. Particles of these sizes are theoretically adequate for anticancer drug delivery applications.

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