LIPID NANOSTRUCTURES AS OPTIMAL CARRIERS FOR ENCAPSULATION OF BIOACTIVE COMPOUNDS

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The paper aims at obtaining lipid nanostructures based on basil oil to be used in the encapsulation process of some bioactive compounds. In order to assure a high degree of homogenization, two adequate techniques have been successively applied: high shear homogenization (HSH) and high pressure homogenization (HPH). The synthesis procedure has been optimized by following four consecutive stages in which a series of relevant parameters have been monitored: working pressure and number of HPH cycles, HSH time exposure, concentration of surfactant, induction period for pre-emulsion formation, and proportion of lipids within lipid mixture. The lipid nanostructures thus obtained will be further used as efficient carriers to encapsulate various ingredients with antifungal action.

Keywords: nanostructure lipid carriers, basil oil extract, high shear homogenization, high pressure homogenization, synthesis parameters

1. Introduction

Of great interest for the scientific community and a key field of pharmaceutical research is represented by the nanotechnology.

The use of this instrument has proved to be optimal, due to specific physical-chemical properties and capacity of nanostructures to overcome several biological barriers in order to be involved in various processes at the cellular level. Thus, there might be possible among other things, the release of therapeutic compounds in the area of interest, within the optimal dosing interval while reducing adverse effects and the toxicity of the active substance and the control of certain biological phenomena at the cellular level, including gene therapy [1-4].

Some of the advantages of using these structures consist in the loading capacity of various types of drugs as compared to other colloidal systems; the matrices are also associated with the controlled release of the drug in the area of interest and with the possibility of improving the formulation regarding the increased adsorption of active substance.

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These advantages and better understanding of the mechanisms and processes involved in the transport of nanoparticles contribute to the maintenance of the high level of scientific and practical interest in order to obtain new nanostructures that could provide other perspectives of application in nanomedicine.

Among the major areas of action in which the research of the nanostructures has an expanding development are the cosmetics and the medicine. Nowadays of topical interest are researches and studies carried out to improve sunscreen and anti-aging products based on squalene [5], the glyceryl monostearate [6, 7], with maintenance and preventive actions, reparative, mitigation, regenerating and revitalizing actions for the skin.

The medicine is the field where nanoscience applications become more and more attractive. As a result, there are studies carried out to obtain some biosensors for the rapid detection of various diseases [8.9], in particular for the early detection of cancer and the monitoring of the homeostasis of the organism. Numerous studies have as objective the obtaining or the improvement of new drugs, vaccines and serums.

The scientific literature presents many studies regarding the loading of active substances in lipid nanocarriers of SLN type (solid lipid nanoparticles) and NLC (nanostructured lipid carriers) with antibiotics (antiviral, antifungal, antibacterial) nystatin [10-12], quercetin [13], itraconazole [14, 15, 26, 27], with anticancer drug : doxorubicin [16, 28], resveratrol [17, 29, 30], etc.

The formulation technologies applied lead to stable physical and chemical properties of the nanostructures, high loading capacity of the active substance, a good release of drug in the selected area in a sufficient amount and in a systematical rhythm and long-term stability of the formulation.

The mixture of fat used in the synthesis of the nanostructured lipid carriers (NLC) contains at the ambient temperature a lipid-liquid part and a lipid-solid part. An advantage of these systems consists in fact that the lipid matrix is composed of physiological lipids that reduce the risk of acute and chronic toxicity.

The solid lipid nanoparticles are made of 0.1% to 30% (w / w) of solid lipid dispersed in an aqueous medium, with size average of the particles ranging between 4 - 1000 nm [18-21].

By increasing the lipid content by more than 10%, particles are being frequently obtained with sizes up to the order of microns and with a large poly-dispersity, phenomena explained by the tendency of agglomeration of particles at high concentrations of lipid.

Studies from the literature show that nanostructure properties are influenced by the lipids from the composition that act on the following

parameters: biocompatibility and toxicity, high loading capacity, solubility of the active substance, drug release, chemical stability, etc. [22-25].

The stabilization of lipid dispersion involves choosing a combination of emulsifiers / surfactants with good efficiency in preventing the agglomeration of particles. Depending on the concentration of lipids, surfactants are added in a proportion of 0.5 - 5% in order to obtain a nanosystem physically stable [31].

These nanostructures of NLC type must not be toxic to the body and should have a good biological activity; the applied dose should be effective in the area of interest, with fungicidal or fungistatic activity.

In this context, a non-saturated oil of natural origin, basil oil extract, has been introduced as active component into the lipid mixture of NLC-type nanostructure in order to enhance the efficiency of a currently used antibiotic and to prevent the adapting capacity and development of fungi resistance to antibiotics.

The objective of this study is associated to establishing some experimental optimal working conditions in order to obtain new nanostructure lipid carriers (NLCs) based on basil oil, with average diameters smaller than 200 nm, stable from the physical point of view and that can be successfully used to encapsulate some active compounds with antifungal properties. In order to assure a high degree of homogenization, two adequate techniques have been successively applied: high shear homogenization (HSH) and high pressure homogenization (HPH), and the synthesis parameters have been selected after a careful monitorization.

2. EXPERIMENTAL

2.1 Materials

In order to obtain the two phases, aqueous and lipid, the following materials have been used:

- Aqueous phase: lecithin (L) - L-α-phosphatidyl choline, and poloxamer (POX) - Synperonic PE/F68 (Poloxamer 188, block copolymer of polyethylene and polypropylene glycol) obtained from Sigma Aldrich Chemie GmbH (Munich, Germany), Twin 80 (Tw 80) – polyoxyethylene sorbitan monooleate purchased from Merck (Germany), distilled water;
- Lipid phase: cetyl palmitate (CP) - obtained from Acros Organics (USA), glyceryl monostearate (MSG) from Cognis GmbH and basil oil extract (UB) from Hofigal Bucharest, NaCl 9% for Zeta potential measurements.

2.2 The synthesis of different lipid structures and characterization techniques

Homogenization process is essential to achieve the quality and stability standards of products. The lipid nanostructures have been obtained by combining two homogenization techniques, one of them providing a high level of shearing - HSH, using a High-Shear Homogenizer PRO 250 device, of 0 - 28000 rpm, 300 W, Germany, and the other to produce a homogenization at high pressure - HPH, using HPH, APV 2000 Lab Homogenizer, Germany. The process of high pressure homogenization leads to the disintegration of the particles and their uniformity depending on the characteristics of the material, the pressure used and the number of homogenization cycles.

The main stages of NLC synthesis are presented in Fig. 1 according to the following schedule.

The first step in obtaining the nanostructure of interest is the optimization of the synthesis conditions that consists in four consecutive stages in which several parameters are controlled in detail:

1. lipid composition in connection with HPH working conditions;
2. surfactant concentration;
3. time of pre-emulsion formation;
4. HSH working time.

The synthesis procedure requires the following conditions:

- 25^{o}C ambient temperature;
- the concentration of surfactant is constant, 2%, with a ratio of 80% Tw 80, 10% L, 10% POX among the components of surfactant mixture;
- the variation of the lipid concentration while maintaining a constant ratio among the components of the lipid mixture: 45% MSG, 45% PC and 10% UB.

To work at HPH are required:

- 100 g of sample containing 2% surfactant, x% fat and filled with distilled water up to 100%.

The aqueous and lipid phases are heated at a temperature of ~ 90^{o}C. Add the lipid phase when lecithin from aqueous phase liquefies. The samples are heated and agitated simultaneously in order to shorten the exposure time at a high temperature and for a proper mixing.

When the lipids liquefy and the mixture become homogeneous, the two phases are mixed obtaining the pre-emulsion. This is stirring for 30 minutes in a water bath at a temperature of ~ 90°C. The pre-emulsion is processed by HSH for 1 minute at 10,000 rpm. At the same time the HPH tank is heated to a temperature above 80°C with distilled water. After 1 minute add the sample in HPH vessel,

establish working pressure and start the working cycles. The process is interrupted every cycle and a sample is collected.

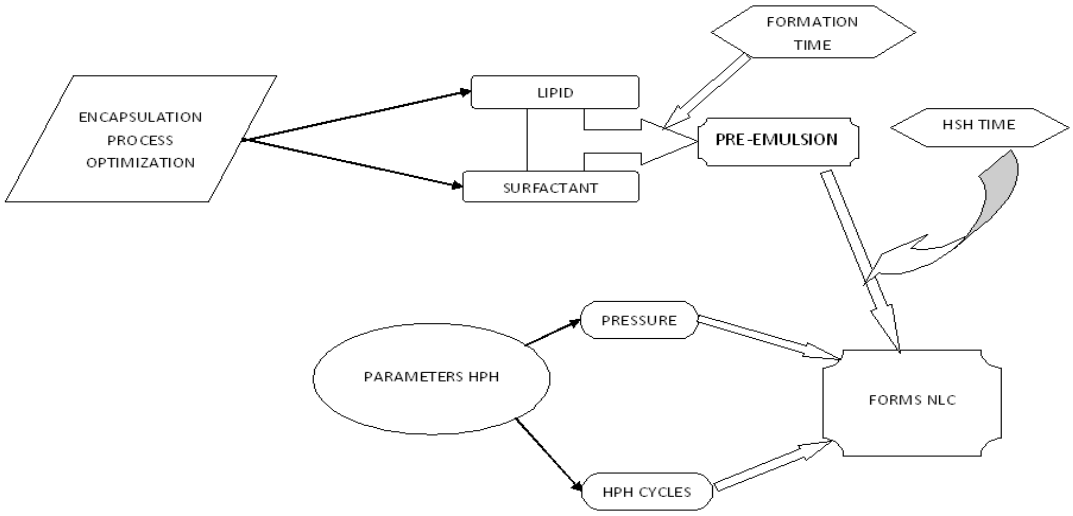

Fig. 1. The main steps of NLC synthesis

Three parallel samples of each lipid concentration are weighted and they are exposed to 500 bar, 600 bar and 800 bar. At every 3 HPH cycles a sample of 100 μg is collected with an automatic pipette in order to measure the particle size and zeta potential, up to 12 HPH cycles.

In order to measure the particle size, 100 μg of sample, collected with an automatic pipette is added in a conic flask with 25 mL of distilled water and is gently mixed. The dynamic light scattering (DLS) hopper is filled with a micro-pipette and the size particle and poly-dispersity are determined. 60 μL of 9%, NaCl are added in the conic flask, homogenised by mixing and the zeta potential is measured by DLS.

2.3 Characterisation methods

Zeta potential (ZP) was determined by measuring the electrophoretic mobility of nanoparticles in an electric field, using the Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK), based on Helmholtz-Smoluchowsky relationship. Before the measurement, the dispersion of lipid nanoparticles were diluted with sodium chloride solution (0.9%, w / v) in order to adjust the conductivity to 50 μS/cm. All measurements were made in triplicate at 25°C, making an average of these measurements.

Particle size measurements were analysed by dynamic light scattering using the same Zeta sizer Nano ZS apparatus. Mean particle size (Zave) and poly-dispersity index (Pdi) have been measured at a diffusion angle of 90° and at the temperature of 25°C. Before these measurements the nanoparticle dispersions were diluted with deionized water to suitable dispersion intensity. The particle size data were evaluated using the distribution of intensity. Average diameter (based on the Stokes-Einstein equation) and poly-dispersity index are given as the average of three measurements.

3. Results and discussion

3.1 Optimization of the concentration of lipid, the number of cycles and the HPH operating pressure

In the table 1 are summarized the working parameters of a number of 10 samples containing 2% surfactant mixture, time of pre-emulsion formation of 30 minutes and time of HSH exposure of 1 minute.

Table 1

Lipid concentration and HPH operating conditions for samples containing 2% surfactant mixture, time of pre-emulsion formation of 30 minutes and time of HSH exposure of 1 minute

Sample code	Lipid phase content (%)	HPH pressure (bar)	HPH cycles
NLC 1_3	10	500	3
NLC 1_6	10	500	6
NLC 1_9	10	500	9
NLC 1_12	10	500	12
NLC 2_3	10	600	3
NLC 2_6	10	600	6
NLC 2_9	10	600	9
NLC 2_12	10	600	12
NLC 3_3	10	800	3
NLC 3_6	10	800	6
NLC 3_9	10	800	9
NLC 3_12	10	800	12
NLC 4_3	7	500	3
NLC 4_6	7	500	6
NLC 4_9	7	500	9
NLC 4_12	7	500	12

Sample code	Lipid phase content (%)	HPH pressure (bar)	HPH cycles
NLC 5_3	7	600	3
NLC 5_6	7	600	6
NLC 5_9	7	600	9
NLC 5_12	7	600	12
NLC 6_3	7	800	3
NLC 6_6	7	800	6
NLC 6_9	7	800	9
NLC 6_12	7	800	12
NLC 7_3	15	500	3
NLC 7_6	15	500	6
NLC 7_9	15	500	9
NLC 7_12	15	500	12
NLC 8_3	12	500	3
NLC 8_6	12	500	6
NLC 8_9	12	500	9
NLC 8_12	12	500	12
NLC 9_3	12	600	3
NLC 9_6	12	600	6
NLC 9_9	12	600	9
NLC 9_12	12	600	12
NLC 10_3	12	800	3
NLC 10_6	12	800	6
NLC 10_9	12	800	9
NLC 10_12	12	800	12

The mixture proportions of surfactants (Tw, L and POX) and the working concentrations related to optimization stage have been established based on the literature data and our previous expertise [6, 7, 13, 16, 17, 19, 32].

The lipid concentration was ranging between 7 - 15%, while maintaining a constant ratio between the components of the lipid mixture.

In order to optimize the number of working cycles and the pressure, samples of various concentrations were subjected to pressures of 500, 600 and 800 bar. To determine the number of HPH cycles, 100 μg of sample were collected at every three cycles and DLS measurements were performed aiming at determination of zeta potential, the particle size and poly-dispersity.

The results obtained for the concentration of 10% lipid phase show that at a pressure of 500 bar particles are obtained with size between 200 and 250 nm and a high poly-dispersity (0.328 - 0.482) as shown in Fig. 2, which suggests that these structures are not appropriate. At a pressure of 600 bar, the size of particles obtained is ranging from 150 nm to 175 nm with good Pdi below 0.18 for 6 or 9 HPH working cycles and good stability (ZP = - 42 mV). At the pressure of 800 bar, particle size remains in the same size range, but the Pdi increases resulting into lower homogeneity, although the structures are stable (ZP: -41, -50 mV).

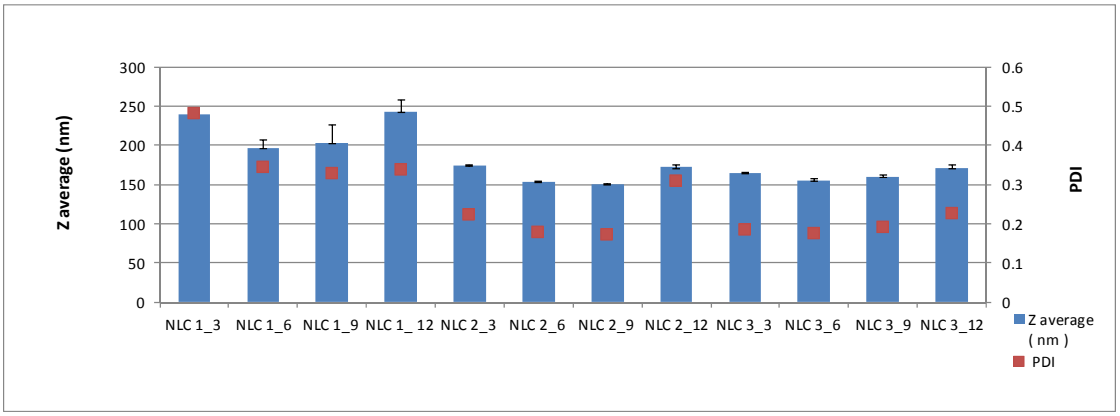

Fig. 2. Variation of size and poly-dispersity function of pressure and cycle number for 10 % lipid phase structure

For 7 % lipid phase and pressures of 500 and 800 bar lower particle sizes have been obtained (130 - 180 nm) by using 6 and 9 cycles, while Pdi is over 0.21, meaning low homogeneity degree (Fig. 3).

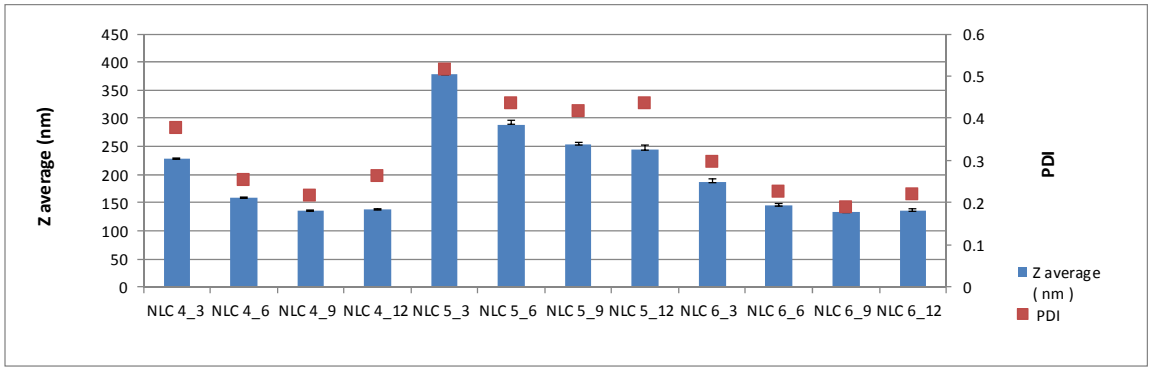

Fig. 3. Variation of size and poly-dispersity function of pressure and cycle number for 7 % lipid phase structure

For concentrations of 15% of the lipid phase, the particle size of more than 240 nm were obtained and a Pdi over 0.3 which suggests that these conditions are not optimal for the synthesis of the desired product. It also appears that beyond a certain limit an increase in the concentration of lipid phase results in increase of the particle size and the aggregation phenomenon, observation supported by data presented in the literature [13, 19].

For concentrations of 12% of the lipid phase the optimal results have been obtained (Fig. 4) with a particle size in the range of 150 - 170 nm for working pressures of 600 and 800 bar, and 6 or 9 HPH working cycles. The best Pdi (0.156) was obtained for a total of 9 HPH working cycles.

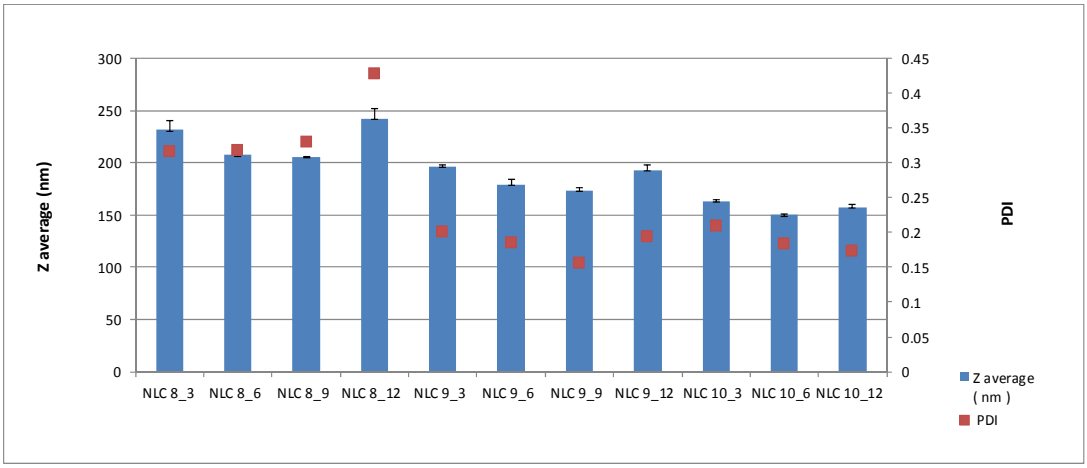

Fig. 4. Variation of size and poly-dispersity function of pressure and cycle number for 12 % lipid phase structure

The experimental data presented above show that the particle size decreases while the cycle number increases (up to 9 cycles). A further increase in the number of working cycles up to 12 HPH cycles leads to an increase in the size of the particles and of the Pdi and a dramatically decrease in the stability of the structures.

Based on these experimental results two optimal lipid concentrations were obtained (10% and 12%) for the pre-emulsion mixture, optimal pressure of about 600 - 800 bar, and an optimal number of 6 - 9 HPH working cycles.

Keeping constant the optimal parameters established above (HPH pressure of 800 bar, 6 HPH cycles and 10 % lipid phase) in the following experiments have been systematically varied the surfactant concentration, time of pre-emulsion formation, and HSH exposure time (table 2).

Table 2

Samples with various concentrations of surfactant mixture, time of pre-emulsion formation and time of HSH exposure at constant HPH parameters and lipid phase content

Sample code	Concentrations of surfactant mixture (%)	Time of pre-emulsion formation (min.)	Time of HSH exposure (min.)
NLC 11	1	30	1
NLC 12	1.5	30	1
NLC 13	2	30	1
NLC 14	2.5	30	1
NLC 15	3	30	1
NLC 16	3.5	30	1
NLC 17	2.5	15	1
NLC 18	2.5	30	1
NLC 19	2.5	45	1
NLC 20	2.5	60	1
NLC 21	2.5	45	0
NLC 22	2.5	45	1
NLC 23	2.5	45	2
NLC 24	2.5	45	3

3.2 Optimization of surfactant concentration

For the second stage, the operating conditions of temperature (25°C), pressure (800 bar), HSH working time (1 minute), the time of formation of the pre-emulsion (30 min), the number of HPH working cycles (6 cycles) and the ratio of the components in the two phases: aqueous and lipid have been maintained constant. The working concentration was 10% of the mixture of lipid, the concentration of surfactant ranging from 1 to 3.5% (Fig. 5).

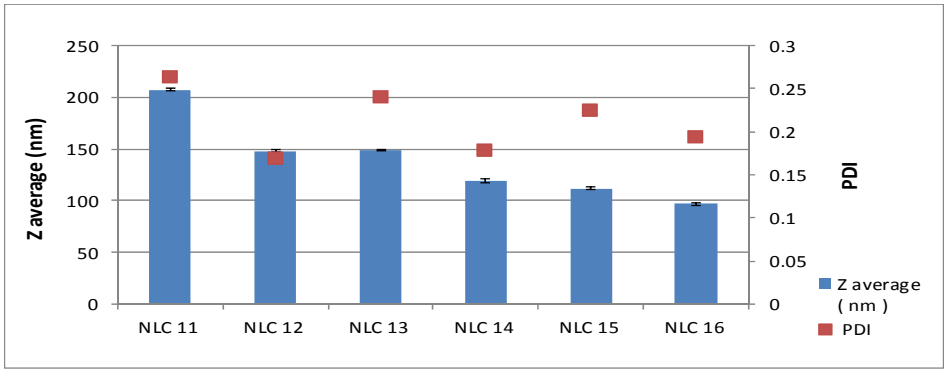

Fig. 5. Variation of the particle size and poly-dispersity function of the surfactant concentration

One can observe that the particle size decreases with increasing surfactant concentration and a good stability of the particles was obtained (ZP = 24.5 - 33.6 mV); the best Pdi (0.169; 0.178) was obtained for a mixture of surfactant of 1.5% and 2.5% and even for NLC with 3.5% mixture of surfactant (0.194). Moreover, the increase by one unit from 1.5% to 2.5% of the concentration of the surfactant mixture results in a significant decrease in particle size from 148.1 nm to 118.8 nm. Further increase by one unit until 3.5% in the proportion of the mixture of surfactant leads to the NLC sizes below 100 nm (~ 97 nm) but with a lower stability of the formulation, ZP < 25 mV.

However, given that the literature data show that a higher concentration of surfactant might have toxic effects on the body [33, 34, 35] and by corroborating with our results obtained on particle size and Pdi, it was established that optimal working concentration is 2.5 % surfactant mixture.

3.3 Optimization of pre-emulsion formation time

The working conditions from previous stage are preserved and the optimal surfactant concentration of 2.5% has been chosen, while the pre-emulsion formation time was varied from 15 to 60 minutes (Fig. 6).

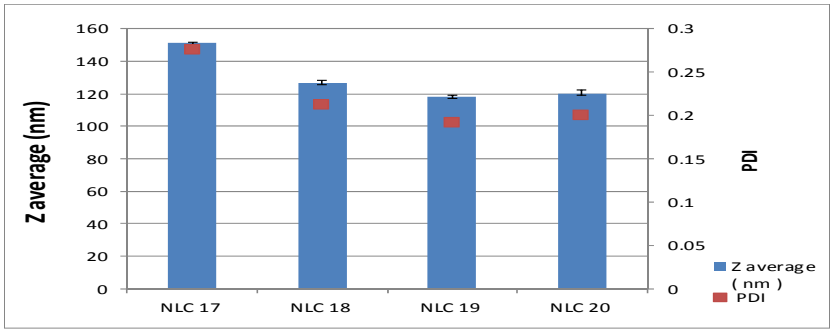

Fig. 6. Variation of particle size and poly-dispersity function of pre-emulsion formation time

One can notice that by increasing the time of pre-emulsion formation up to 45 minutes, the particle size decreases and the Pdi decreases as well. At longer period of formation, particle size and Pdi are increasing, while the stability is drastically decreasing (ZP= -0.143 mV). For a formation time between 30 and 45 minutes are obtained NLC with similar size and Pdi, 126.3 nm or 118.2 nm and 0.112 to 0.191 respectively, but the stability is better for the structures formed in 30 minutes than those formed in 45 minutes (25 mV as compared to 20 mV). As a result, during the following procedures the time of 30 minutes has been used to form the pre-emulsion.

3.4 Optimization of HSH working time

For the fourth stage the working conditions from previous stage are preserved, selecting the pre-emulsion formation time (30 min) and HSH exposure time ranging from 0 to 3 minutes.

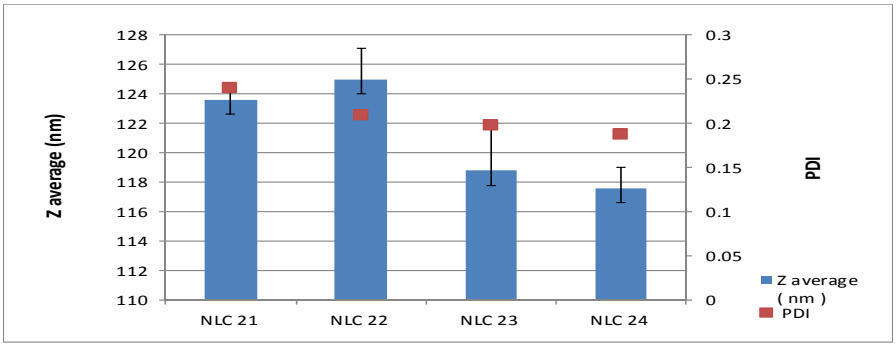

Fig. 7. Variation of particle size and poly-dispersity function of HSH working time

One can see that increasing HSH exposure time leads to progressive decrease in particle size and Pdi (Fig. 7). For exposure of 2 and 3 minutes the results are quite similar (118.8 nm and 117.6 nm) but the stability is better for 2 minutes HSH exposure (ZP = -24.8 mV).

As a result, the optimum HSH working time for the next syntheses is considered to be 2 minutes.

4. Conclusion

By coupling the two techniques, high shear mixing and high pressure homogenization, stable lipid nanostructures based on basil oil have been obtained, with dimensions in the range of 120 - 170 nm.

Optimization of the synthesis procedure of such nanostructures was performed in four steps according to the following working parameters:

- an optimum number of 6 - 9 cycles working at HPH, having a high stability;
- an optimum surfactant concentration of 2.5% ;
- the optimal formation period of the pre-emulsion in the range of 15 - 45 min;
- the optimum working time at HSH in the range of 1 - 2 min;
- NLC type structures synthesized with 10% lipid phase and 12% lipid phase, having optimal dimensions and a very good stability.

This parameter optimization is the first step in the process of obtaining the NLC of interest, such lipid nanostructures being further used as efficient carriers to encapsulate various ingredients with antifungal action.

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