New Approaches on the Synthesis of Effective Nanostructured Lipid Carriers

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Lipid based particles such as solid lipid nanoparticles and nanostructured lipid carriers are among the most promising carrier systems used in many domains, e.g. medicine, cosmetics and food industry. The aim of this study was to evaluate the influence of various kinds of solid lipid matrices on synthesis of effective nanostructured lipid carriers with appropriate average diameters and physical stability. For this purpose two solid lipid mixtures (glycerol monostearate with carnauba wax and glycerol monostearate with beeswax) in combination with three kinds of vegetable oils enriched in w-3, -5, -6 and -9 fatty acids (e.g. raspberry oil, rice oil, pomegranate oil) have been used for preparation of lipid nanocarriers. The free lipid nanocarriers and ivy leaves extract loaded nanocarriers were synthesized by high shear homogenization coupled with high pressure homogenization. The effect of surfactants mixture and loading with vegetable mixture has been followed by dynamic light scattering, electrokinetic potential measurements and differential scanning calorimetry.

Keywords: nanostructured carriers, solid lipids, high pressure homogenization, physical stability

Solid lipid nanoparticles (SLN), introduced in 1991, are spherical particles with average diameter in the nanometer range, consisting of a solid lipid matrix core that can solubilize the lypophil drugs [1-3]. The lipid core is stabilized by physiologically compatible surfactants and co-surfactants (with concentrations between 0.5-5%) such as polysorbates, polyoxyethylene ethers, phospholipids, bile salts and so on [2]. Nanostructured lipid carriers (NLC) are the second generation of lipid based nanoparticles developed at the begining of 2000 [4]. NLC are obtained by replacing a part of the solid lipid used in the SLN formulation by a liquid lipid, in order to reduce the ordered structure of the lipid matrix and to introduce imperfections necessary for minimizing the drug expulsion upon storage [5, 6]. Nanostructured lipid carriers, similar to the SLN, are systems with solid lipid matrix at body temperature [7, 8]. Incorporation of liquid lipid inside the lipid core of nanostructured lipid carriers leads to a massive disruption of the crystalline arrangement [9, 10], unlike solid lipid nanoparticles (SLN) that form a perfect crystal [11, 12]. Depending on the composition and on the preparation procedures, the size and structural properties of the lipid particles vary, typically the main diameters of SLN and NLC ranging between 50 and 400 nm [13, 14].

Both kinds of lipid nanoparticles, SLN and NLC, exhibit features attributed to physico-chemical phenomena mainly associated with the physical state of the lipid phase [15-17]. *Firstly,* the mobility of drugs in a solid matrix is lower than in a liquid matrix. This leads to an improved stability of labile active ingredients [18-20]. Moreover, the microphase separations of the active ingredients and carrier lipid within individual particles can be controlled, thereby preventing the accumulation of active compounds at the surface of lipid particles where chemical degradation reactions often occur [21-23]. *Secondly*, incorporation of poorly absorbed bioactive compounds into solid lipid nanoparticles has been shown to improve their biological absorption [24, 25]. *Thirdly,* lipid nanoparticles are able to provide a carrier system with controlled release properties [26, 27].

The main objective of this study was to evaluate the influence of various kinds of solid lipid matrices on synthesis of effective nanostructured lipid carriers with appropriate average diameters and physical stability. For this purpose two solid lipid mixtures (glyceryl monostearate with carnauba wax and glycerol monostearate with beeswax in combination with natural oils of vegetable origin have been chosen, in order to create high disordered crystal lattice able to accommodate a complex mixture of vegetable extract (e.g. ivy leaves extract). As liquid lipid, different vegetable oils rich in omega fatty acids with different structural characteristics have been used (e.g. raspberry oil concentrated essential fatty acid omega-3, rice oil containing a different fatty acid omega-6 and omega-9, pomegranate oil concentrated in omega-5). The free NLC and ivy leaves extract loaded NLCs were prepared by high shear homogenization coupled with high pressure homogenization. The nanostructured lipid carriers were characterized in terms of dynamic light scattering, electrokinetic potential measurements and differential scanning calorimetry.

Experimental part

Materials and methods

Polyoxyethylenesorbitan monolaurate (Tween 20) was purchased from (Lyon, France), Glycerol (Gly) was obtained from (Belgium). Poloxamer 407 (block copolymer of polyethylene and polypropylene glycol) was supplied by (Burgbernheim, Germany) and Soybean lecithin from (Hamburg, Germany). Carnauba wax (CW) and Beeswax (BW) was obtained from (Trittau, Germany) and Glycerol Stearate (GS) from (Monheim, Germany). Raspberry oil (RaO), Rice oil (RO) and Pomegranate oil (PO) was obtained from (Oradea, Romania). Sodium cholate was supplied by (New Zealand) and dry extract of ivy leaves (ILE) was supplied by (Germany).

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Type of formulation[1]	ILE[2] %	Mixed surfactants[3]	Vegetable oil
Set I. Carnauba wax (CW) : Glycerol Stearate (GS)			
NLC-1			RO
NLC-2	0		PO
NLC-3			RaO
NLC-ILE-1		Tween 20/	RO
NLC-ILE-2	0.1	Soybean	PO
NLC-ILE-3		lecithin/	RaO
NLC-ILE-4		Poloxamer 407	RO
NLC-ILE-5	0.2		PO
NLC-ILE-6			RaO
NLC-4			RO
NLC-5	0		PO
NLC-6		Sodium cholate/	RaO
NLC-ILE-7		Tween 20/	RO
NLC-ILE-8	0.1	Poloxamer 407	PO
NLC-ILE-9			RaO
NLC-ILE-10			RO
NLC-ILE-11	0.2		PO
NLC-ILE-12			RaO
Set II. Beeswax (BW) : Glycerol Stearate (GS)			
NLC-7	0		RO
NLC-8		Tween 20/	RaO
NLC-ILE-13	0.1	Soybean	RO
NLC-ILE-14		lecithin/	RaO
NLC-ILE-15	0.2	Poloxamer 407	RO
NLC-ILE-16			RaO
NLC-9	0		RO
NLC-10		Sodium cholate/	RaO
NLC-ILE-17	0.1	Tween 20/	RO
NLC-ILE-18		Poloxamer 407	RaO
NLC-ILE-19	0.2		RO
NLC-ILE-20			RaO

Table 1
THE COMPOSITION OF NLC FORMULATIONS

[1]All NLCs formulations have been prepared with 10 % (w/w) lipids mixture, in a ratio 70% of GS:CW = 1:1/ GS:BW = 2.5:1 and 25% RO/ RaO/ PO and 5% Gly
[2]The initial concentration of dry extract of ivy leaves
[3]All samples were prepared using 2.5% mixed surfactants Tween 20, Soy Lecithin, Poloxamer 407 (with a mass ratio of 16:1:3)/ Sodium cholate, Tween 20, Poloxamer 407 (with a mass ratio of 40:9:1)

Preparation of nanostructured lipid carriers

Briefly, in a melted lipid phase composed of GS, CW/ BW, RO/ RaO/ PO, appropriate amount of ILE has been added. The lipid mixture has been kept for five minutes before mixing with an aqueous surfactants phase. The hot emulsion resulted by mixing of lipid and aqueous phase at 85°C was submitted to an external mechanical energy by high shear homogenization with a Lab rotor-stator Homogenizer (High-Shear Homogenizer SC 250 type; 0~30.000 rpm; power of 250 W, PRO Scientific, USA), by applying 10 000 rpm for 2 min. Then, hot emulsion obtained is subjected to high pressure homogenization (6 cycles at 800 Barr) by using an APV 1000 Invensys Homogenizer (Denmark). The obtained emulsion was allowed to cool down at room temperature, with formation of aqueous NLC dispersions. The composition of each NLC formulation is presented in table 1. Finally, the NLCs dispersions were submitted to a lyophilization process (-55°C for a period of 72h), by using an Alpha 1-2 LD Freeze Dry System equipment (Germany), in order to obtain powders of NLCs.

Characterization of nanostructured lipid carriers

Particles size analysis

Particle size measurements were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS. The mean particle size (Z_{av}) and the polydispersity index (PdI) of the NLC dispersions were measured at a scattering angle of 90° and at a temperature of 25°C. Before measurements, the dispersions were diluted with deionized water to an adequate scattering intensity. The particle size data were evaluated using intensity distribution. The average diameters (based on Stokes-Einstein equation) and the polydispersity index were given as average of three individual measurements.

Zeta potential analysis

The Zeta potential (ZP) was determined by measuring the electrophoretic mobility of the nanoparticles in an electric field, by using the Helmholtz-Smoluchowsky equation. Prior to the measurements, taken with the appropriate accessory of Zetasizer Nano ZS, the nanostructured lipid carriers in dispersion were diluted with a sodium chloride solution (0.9%, w/v), to adjust the conductivity to 50 μS/cm. All measurements were performed at 25°C, in triplicate and the mean value was reported.

Differential scanning calorimetry (DSC)

The changes in the crystalline states of the lipid matrix of lyophilized free- and WBE loaded-NLCs were studied by differential scanning calorimetry. The DSC analysis was performed using a Jupiter, STA 449C differential scanning calorimeter. The samples (10 mg) were weighed into standard alumina pans. An empty pan was used as reference. The thermal analysis profiles were obtained as the temperature was increased from 30 to 100°C at a rate of 5 °C/min.

Results and discussions

Two sets of lipid nanostructures have been synthesized (table 1), by using a mixture of solid lipids as: glyceryl monostearate in combination with carnauba wax or glyceryl monostearate and beeswax. For each set, three types of vegetable oils have been used alternatively as natural liquid fat: rice oil (RO), pomegranate oil (PO) and raspberry oil (RaO). For each NLC formulation a concentration of 2.5% surfactants and co-surfactant mixtures was selected (e.g. Tween 20/Soya lecithin/ Poloxamer 407 and Sodium cholate/Tween 20/Poloxamer

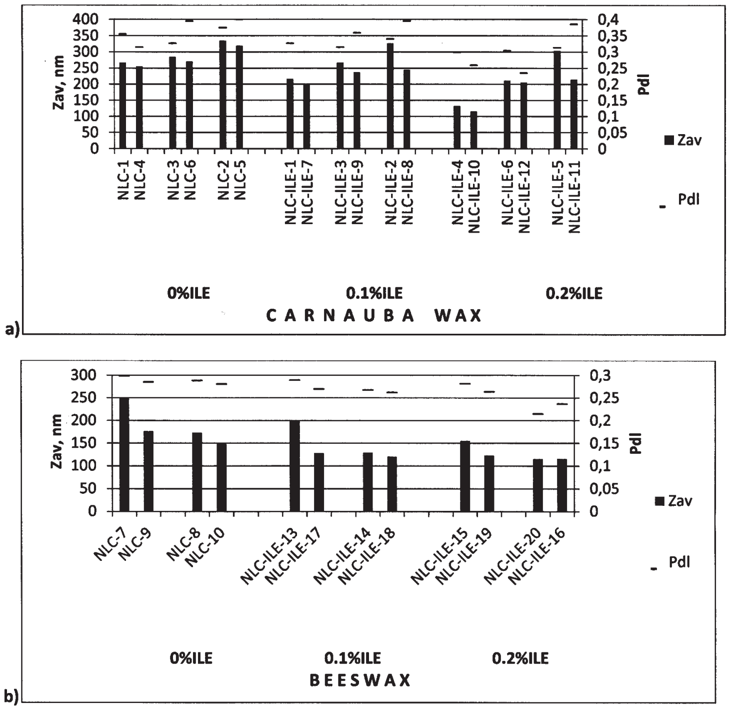


Fig. 1. Influence of the surfactants mixture on the particle size and polydispersity index:
a. lipid matrix synthetized with carnauba wax;
b. lipid matrix synthetized with beeswax

407). To facilitate solubilization of dry extract of ivy leaves in the complex lipid core, an additional co-surfactant - Glycerol has been used. In order to exclude the influence of glycerol on Zav and PDI in glycerol content was kept constant in all samples synthesized (NLC free and NLC loaded with ivy leaves extract).

Particles size analysis of free- lipid nanocarriers and loaded with a vegetable extract

The effect of surfactants mixture and lipid matrix on Zav and PDI.

From the results obtained (fig. 1a), it can be seen that the use of the mixture containing Tween 20 as the main surfactant, in association with the lipid mixture consisting of carnauba wax, glycerol monostearate and vegetable oil has resulted in obtaining particles with average diameter between 211.5 ÷ 334.5 nm. Good results have been also obtained for particle size in the case of using the mixture of surfactants with Sodium cholate as the main surfactant (115.6 ÷ 319.6 nm). The decrease of particle size can be associated with the presence of ionic surfactant (sodium cholate), which gives better coverage of the lipid core resulting in its better compacting. With regard to the polydispersity index, the distribution of nanocarriers prepared with these surfactants is relatively high (above 0.3). These values reveal the existence of populations of lipid particles of various sizes. To overcome these shortcomings, by means the obtaining of NLC with low degree of polydispersity, a significant change in the lipid core has been achieved. Thus, carnauba wax was completely replaced by another wax, e.g. beeswax (because waxes are resistant to oxidation and microbial attack), and the content in the fat core was reduced to 20%.

The best results were obtained for particle size in the case of using the mixture of surfactants with sodium cholate as the main surfactant (fig. 1 b). These results can be explained on the basis of complex structures of lipids (solid and liquid) which prefer a surfactant with a less branched structure because of steric hindrance that appears in the solid particle. As reffering to the polydispersity index, the NLCs formulations have similar values for both surfactant mixtures, with values ranging between 0.159 ÷ 0.368.

By using both mixtures of surfactants, in association with the lipid mixture consisting of beeswax (fig. 2), glycerol monostearate and vegetable oil led to significantly lower mean diameters as compared to those of the lipid mixture consisting of carnauba wax, glyceryl monostearate and vegetable oil. Moreover, the polydispersity index significantly decreased, the corresponding lipid nanocarriers having PDI ranged between 0.215 and 0.299.

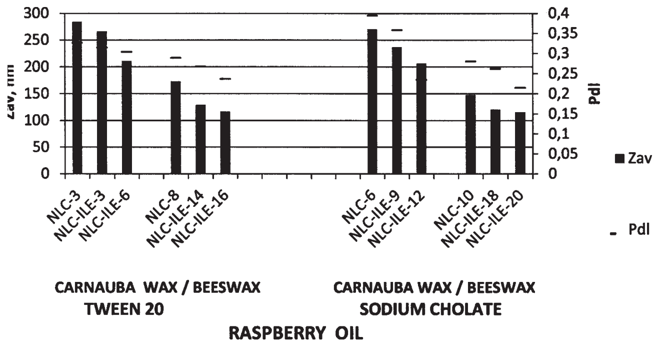


Fig. 2. Influence of the wax lipid matrix type on the particle size and polydispersity index

The influence of vegetable oil type on Zav and PDI

By comparing the effectiveness of three types of vegetable oils selected for the first set of samples (lipid matrix prepared with carnauba wax, fig. 3 a), it can be seen that lipid nanostructures prepared with PO have the highest average diameter size, while the NLC prepared with RO have the smallest value. The difference between the average diameters (Zav) obtained for the NLC prepared with the same solid lipids (set I), but with different vegetable oils may be attributed to the different composition of these oils. Vegetable oils are lipid mixtures with various fatty acids with carbon chains of different lengths.

For the second set of NLCs formulation (lipid matrix prepared with beeswax), from the figure 3 b, it can be observed that the lipid nanostructures prepared with RO have the highest average diameter size. Polydispersity index presents fairly close values for all selected vegetable oil, with values below 0.3.

The efficiency of the vegetable oil could be associated with different structures and compositions of the lipid

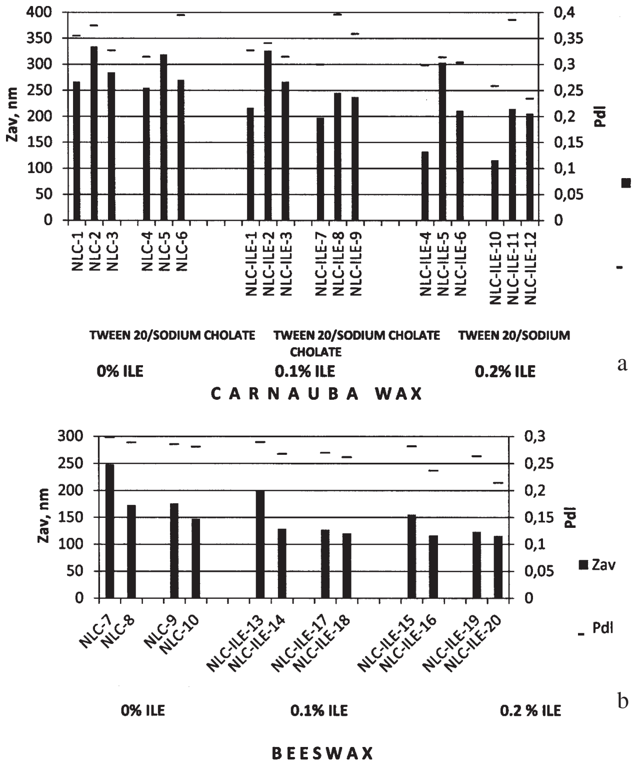


Fig. 3. Influence of the vegetable oil on the particle size and polydispersity index: a. lipid matrix synthetized with carnauba wax; b. lipid matrix synthetized with beeswax

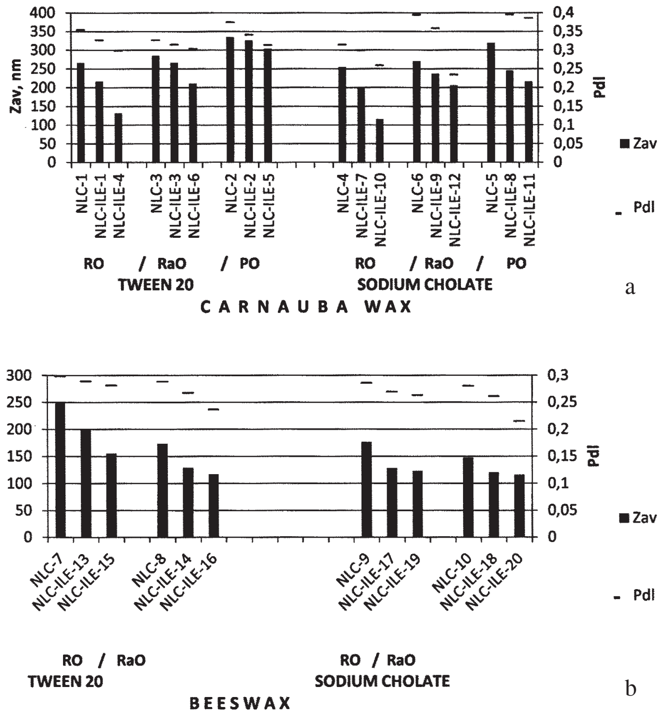


Fig. 4. Variation of particle size and polydispersity index with the increasing of the ILE amount: a. lipid matrix synthetized with carnauba wax; b. lipid matrix synthetized with beeswax

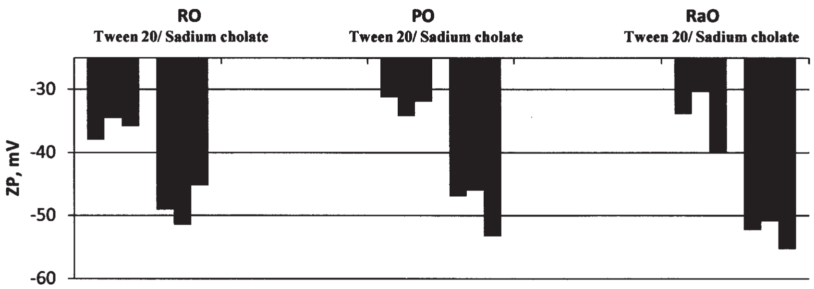


Fig. 5. The effect of surfactant mixture on the physical stability of NLC aqueous dispersions synthesized in the carnauba wax : glycerol stearate system

matrix which may influence the compactation of the lipid core by the mixture of surfactants.

The effect of ivy leaves extract encapsulation on Zav and PDI

By increasing the loading amount of ivy leaves extract, from 0% to 0.1% and 0.2% for all synthesized lipid matrix (fig. 4 a, b), there was a clear decrease of the average diameter of the NLC loaded with ivy leaves extract. This significant decrease in the average particle diameter observed by increasing the plant extract loading could be assigned to the complex mixture of constituents of vegetable extract (for example, carbohydrates), that could lead to a better organization of the lipid matrix. In the NLC-ILE formulation, the complex lipid phase (which represents 10% in the aqueous dispersion) consisting of beeswax (20%), glycerol monostearate (50%), glycerol (5%) and raspberry oil (25 %) proved to be most effective for encapsulation of vegetable extract. For this formulation, using sodium cholate as main surfactant, the lowest average particle diameter (115.6 nm) has been obtained.

Physical stability of the aqueous dispersion of NLC prepared with various lipid matrix

Stability analysis of lipid nanodispersion revealed that all NLC formulations showed a negative electrokinetic potential, with values ranging from - 30.4 ÷ - 64.0 mV (table 1). These results evidenced that the nanocarriers prepared with various kinds of solid lipids and vegetable oils are carriers systems with a strong physical stability.

From the figure 5, it is observed that the type of vegetable oil does not influence the value of zeta potential. Also, the amount of vegetable extract has no effect on the zeta potential value. For the NLC formulations prepared with surfactants mixture that contain Tween 20 as the main surfactant, the zeta potential is from -30 to -40 mV, unlike the formulations prepared with surfactants mixture with sodium cholate as the main surfactant that present strongly electronegative potentials (e.g. -40 ÷ -50 mV). These last values underline a greater physical stability of NLC synthesized with sodium cholate as main surfactant.

As reffering to the effect of the type of wax on the physical stability, it could be observed that NLC made with beeswax (fig. 6) present higher zeta potential values as compared to the NLC made with carnauba wax. For instance, NLC-ILE prepared with beeswax has ZP = - 63.9 mV, while NLC-ILE prepared with carnauba wax has zeta potential value of - 51.5 mV.

As result, the nanocarriers synthesized with beeswax and sodium cholate as main surfactant, exhibit the highest physical stability (ZP = - 63.9 mV), unlike the NLC synthesized with carnauba wax and Tween 20 (as main surfactant), which has the lowest stability, with zeta potential values close to the stability area limit (ZP = - 34.5 mV).

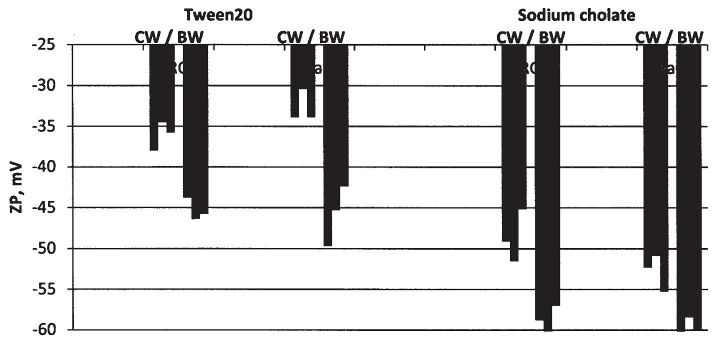
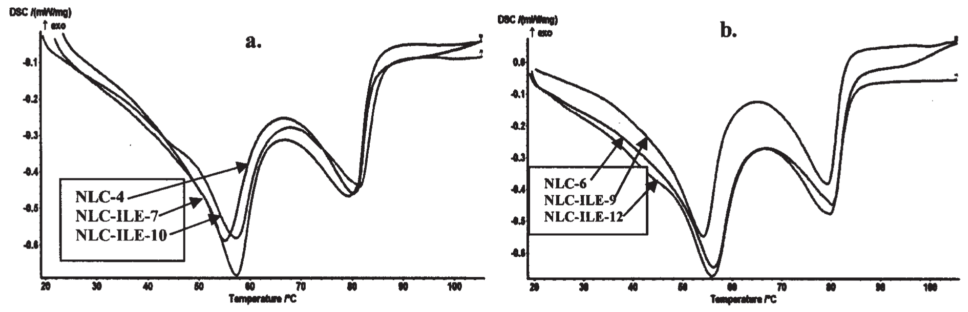


Fig. 6. Effect of the wax type on the stability of NLC aqueous dispersions



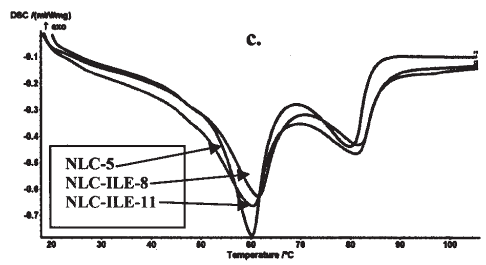
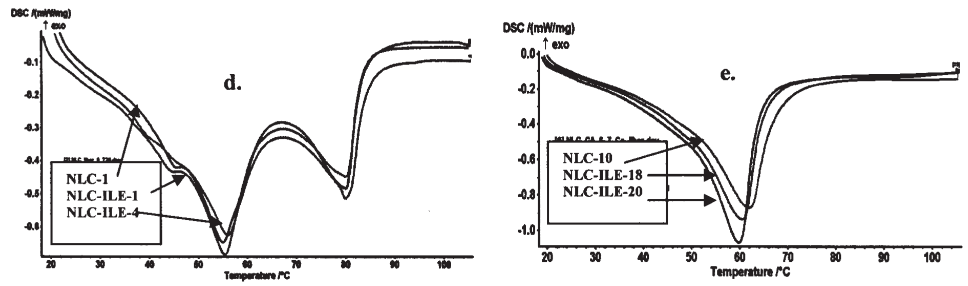


Fig. 7. Differential scanning calorimetry analysis for lyophilized free-NLC and ivy leaves extract loaded-NLC, prepared with: a) rice oil; b) raspberry oil; c) pomegranate oil; d) rice oil; e) raspberry oil



Evaluation of structural changes in lipid matrices after ivy leaves extract encapsulation

Evaluation of the crystal structure of the lipid nanostructures synthesized with various vegetable oils (RO, RaO, PO), different main surfactants (Tween 20 and Sodium cholate), and various waxes (CW and BW), before and after loading with ivy leaves extract, was made based on differential scanning calorimetry (fig. 7).

The results obtained by DSC analysis showed that the degree of lipid crystallization, the melting points and the polymorphism of the free NLC were not influenced by the type of vegetable oil, except in case of nanocarriers loaded with ILE when an obvious disturbance of lipid network has been detected. Thus, both rice and raspberries oils associated with the presence of 0.1% and 0.2% ILE has led to a significant change in the crystalline arrangement, more evident for the NLC loaded with 0.1% ILE. Instead, pomegranate oil associated with ILE led to changes in the crystalline arrangement for both concentrations of extract.

By comparing the DSC curves of NLC formulations prepared with both kinds of waxes it may be observed that NLC synthesized with beeswax has a single endothermic peak and a higher melting temperature. Beeswax in association with ILE decreased the melting temperature and has lead to a widening of endotherm peak, which result in a decreasing of crystalline arrangement.

Conclusions

Using three types of vegetable oils, with various concentrations in omega-3, omega-5, omega-6 and omega-9 fatty acids (RO, PO and RaO) in association with two kinds of solid lipids mixture has resulted in obtaining of stable lipid nanocarriers able to encapsulate a complex plant extract - ivy leaves extract.

By DLS measurements it was observed that the efficiency of vegetable oil may be associated with the matrix composition and lipid structures. Rice oil in combination with carnauba wax and glyceryl monostearate has lead to average size particles lower than 200 nm, while the raspberry oil in combination with beeswax and glyceryl monostearate, showed the lowest average diameter. The composition of plant extract togheter with the surfactants efficiency contributed to a better organization of the lipid matrix, resulting in decrease of average diameter with increasing of ILE amount.

Electrokinetic potential values are electronegative for all NLC formulations with values between - 30.4 ÷ - 64.0

mV, demonstrating excellent physical stability and a low probability of aggregation over time.

The calorimetry study has shown the appearance of some changes in the lipid matrix caused by the presence of ILE (e.g. melting point, lipid crystalinity).

Beeswax used in obtaining the lipid matrix has a dual role: provides better compatibility between glyceryl monostearate and vegetable oils and confers important biological properties (e.g. anti-inflammatory, healing, antibacterial, preservative, emollient, improves blood circulation).

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