Cryoprotector effect on main properties of lipid nanoparticles loaded with bio-active compounds

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The paper is devoted to the investigation of influence of mannitol and trehalose used as cryoprotectors on the main properties of lipid nanoparticles loaded with two kinds of active compounds: an UV molecular absorber (octocrylen) and a natural antioxidant (carotene). The lipid nanoparticles loaded with the selected active compounds were characterized for their physical stability (electrokinetic potential measurements) together with evaluation of size distribution and polydispersity index, by using the dynamic light scattering technique. The crystallization occurred in the inner core of lipid nanoparticles was also investigated by differential scanning calorimetry. Appropriate *in vitro* determination methods have been used in order to evaluate the antioxidant and anti-UV properties of lipid nanoparticles. All experimental results have been shown that a good photoprotection effect and improved antioxidant activities could be obtained by using such cryoprotector agents. By determining the ability of carotene – lipid nanoparticles to scavenge free radicals, it has been shown that the presence of trehalose produced a significant effect on the antioxidant behaviour of nanoparticles. Regarding the anti-UV properties, a concentration over than 5% mannitol has lead to a gradually decreasing of SPF values, while 2.5% trehalose have demonstrated an increasing of SPF index.

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Keywords: Lipid nanoparticles, Cryoprotectors, Octocrylen, Carotene, Antioxidant activity, Photoprotective properties

1. Introduction

Solid lipid nanoparticles (SLNs) introduced since 1991 with double purpose, to combine the features and to avoid disadvantages of other colloidal carriers, are considered as alternative carriers of lipophyl drugs to traditional colloidal systems, such as emulsions, liposomes, microemulsions, micro- and polymeric nanoparticles [1-4]. A lipid system related with the SLN are nanostructured lipid carriers (NLCs) produced by mixing solid lipids with spatially incompatible lipids that lead to a solid lipid matrix with a special structure. NLCs are the new generation of oil-in-water nanoparticulate systems (developed since 1999), being considered to be the latest and smartest generation of lipid nanoparticles that possess improved properties of drug loading, modulation of the release profile, and stable drug incorporation during storage [5, 6]. In this study, both kinds of SLN and NLC were prepared in order to establish the influence of mannitol and trehalose used as cryoprotectors on the main properties of some lipophyl active compounds.

A natural antioxidant widely encountered in various vegetable sources (β-carotene, fig. 1a) [7] and a molecular sunscreen that has both UVA- and UVB-absorbing properties (octocrylen, fig. 1b) [8] were selected for encapsulation into lipid nanoparticles.

The cryoprotector agents have two distinct roles in the synthesis of lipid nanoparticles: to protect against destroying the surfactant shell during lyophilisation process [9] and to assure a steric separation of lipid particles that prevents the possibility of aggregation phenomena [10].

SLN and NLC are attractive colloidal carrier systems, suitable for cosmetics and healthy formulations, in general, due to firstly, their benefical effects on skin [11] and secondly because they are based on nontoxic and noniritant lipids [12].

Photoprotection is an essential prophylactic and therapeutic element which is critical in order to avoid exposure to harmful ultraviolet radiation and the injury induced by UV photons in skin, simultaneously with minimising of local adverse effects [13, 14].

In the cosmetic field, the sunscreen agents has been widely used as photoprotective agents for long time ago [15], but their incorporation into lipid nanoparticles has not yet been fully accomplished. In the last years the encapsulation of cosmetic active compounds into lipid micro- and nanoparticles have evolved since the first incorporations of tocopherol acetate [16], ascorbyl palmitate [17], retinol [18], until natural antioxidants such as coenzime Q10 [19] and vitamin E [20], with the aim of achieving new dermal and cosmetic products.

In this context, the present investigation will focus on the study of the behaviour of two active compounds, after encapsulation into lipid nanoparticles of SLNs and NLCs type and in the presence of cryoprotective agents. Their specific properties, meaning the antioxidant and anti-UV (photoprotective index) properties have been characterized by appropriate *in vitro* methods. Moreover, for exploring the potential of SLNs in improving the photostability in mild irradiation conditions, some cosmetic formulations

were developed and evaluated, based on a combination between a cream base with OCT – SLN.

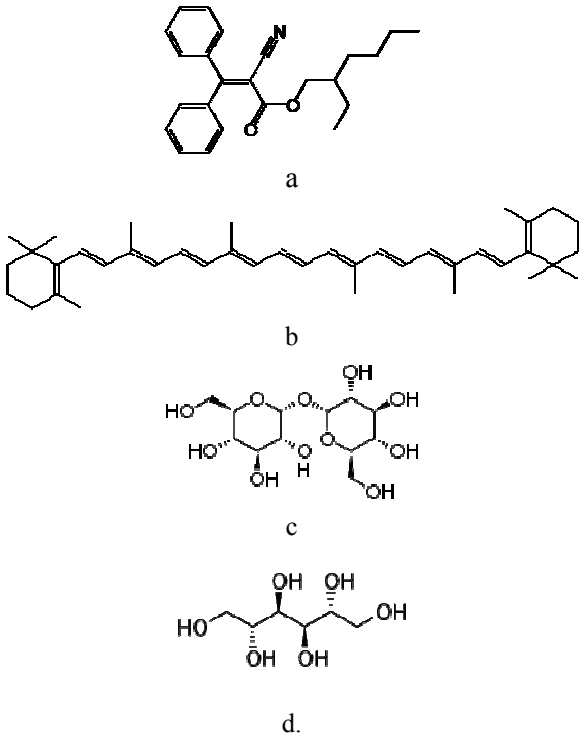


Fig. 1. The molecular structure of active compounds (a. octocrylen and b. carotene) and cryoprotective agents (c. trehalose and d. mannitol).

2. Experimental part

2.1. Reagents

Polyoxyethylenesorbitan monooleate (Tween 80), Polyoxyethylenesorbitan monolaurate (Tween 20), Squalene (SQ), Dimethylsulfoxide and Hydrogen peroxide were obtained from Merck (Germany). Synperonic F68 (block copolymer of polyethylene and polypropylene glycol), L-α-Phosphatidylcholine, (Lecithin), 2-Ethylhexyl-2-cyano-3,3-diphenylacrylate (OCT) and Tris[Hydroxymethyl] aminomethane (Luminol) were purchased from Sigma Aldrich Chemie GmbH. Carotene (>97% purity) was supplied by Fluka Chemie GmbH. n-Hexadecyl Palmitate (CP), 95% was purchased from Acros Organics, USA; Glycerol Stearate (GS), grape seed oil (GSO) and the cream base (which contains stearats, glycerine, fatty alcohols, emulsifier, emollients and an antioxidant – butylhydroxyanisole) were supplied by Elmiplant S.A., Romania.

2.2. Synthesis procedure of octocrylen loaded SLNs and β-carotene loaded NLCs

The lipid nanoparticles were produced by using a combination of melting emulsification with a modified high shear homogenization technique, as was described by authors elsewhere [21]. Briefly, the method involves the existence of two phases: an organic phase that contains the lipid mixture (CP and GS for SLNs preparation and CP, GS and Sq or GSO for NLCs preparation) and active compound (octocrylene and carotene) and an aqueous phase (a surfactant mixture in a weight ratio of Tween : Lecithin : Synperonic F68 = 1:0.25:0.25). Both phases were separately heated at 85^{o}C. In the next step the lipid phase was added into the aqueous surfactant phase and kept to equilibrate at 85^{o}C for 2h. The resulted hot emulsion was further submitted to an external mechanical energy by high shear homogenization with a Lab rotor-stator Homogenizer (High-Shear Homogenizer SAII-20 type; 0~28.000 rpm and power of 300 W, Shanghai Sower Mechanical & Electrical Equipment Co., Ltd, China.), by applying 25 000 rpm for 10 minutes. The obtained emulsion was allowed to cool slowly at room temperature with formation of SLN/NLC dispersions loaded with active compound. In order to remove the water excess and obtain powders of SLNs and NLCs loaded with octocrylen and carotene, respectively, the nanoparticles dispersions were submitted to a liophilization process, by using an Alpha 1-2 LD Freeze Dry System equipment, Germany.

The influence of cryoprotective agents on the main properties of developed loaded-lipid nanoparticles has been achieved by adding different concentrations (2.5 ÷ 10%) of mannitol or trehalose into the aqueous SLN/NLC dispersions, before the lyophilization process. The dispersions have been subsequently freezed at -25^{o}C for 24h, and kept at -55^{o}C for a period of 72h.

2.3. Characterization methods

2.3.1. Particle size analysis

The particle size parameters of lipid nanoparticles given by the hydrodynamic diameters, $z_{average}$ (the particle diameter plus the double layer thickness) and polydispersity index, Pdl (which indicates the width of the size distribution and has a value between 0 and 1) of each SLN/NLC dispersion were determined by using dynamic light scattering technique (Zetasizer Nano ZS, Malvern Instruments Ltd., U.K.), at a scattering angle of 90^{o} and 25^{o}C. Dispersions were analyzed after appropriate dilution with deionised water to an adequate scattering intensity prior to the measurement. The particle size analysis data were evaluated using intensity distribution. The average diameters (based on Stokes-Einstein equation) and polydispersity index were calculated from the three individual measurements.

2.3.2. ξ-potential measurements

The measurement of electrokinetic potential used to assess the charge stability of all SLNs and NLCs loaded with octocrylen or carotene, was realized by applying an electric field across the analyzed aqueous dispersion using the appropriate accessory of Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.). Before zeta potential measurements, all the samples were adjusted with a 0.9%

NaCl solution, in order to correct the conductivity of aqueous dispersions of NLCs at a value of about 55 μS/cm. All measurements were performed in triplicate.

2.3.3. Differential scanning calorimetry

In order to investigate the changes in the crystalline states of the lipid matrix, the lyophilized free- and loaded-SLNs and NLCs were studied by differential scanning calorimetry. The samples (10 mg) were weighed into standard alumina pans. An empty pan was used as reference. Samples were heated at the scanning rate of 5° C/min over a temperature range between 30 and 100 °C. Thermograms were recorded with a differential scanning calorimeter Jupiter, STA 449C (Netzsch).

2.3.4. In vitro evaluation of antioxidant activity

The *in vitro* antioxidant activity of Carotene-NLCs and free-NLCs has been determined by chemiluminescence method (CL) using a Chemiluminometer Turner Design TD 20/20, USA. A cyclic hydrazide (luminol) has been used as light amplifying substance which emits light when oxidized and is converted into an excited aminophtalate ion in the presence of oxidizing species such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1O_2$). H_2O_2 has been used in buffer TRIS-HCl solution (pH = 8.6) as generator system for free radicals. Concentrations of 3.4 μM, 5.4 μM, 7.5 μM and 15 μM carotene solutions were prepared (the sample were submitted to ultrasonic treatment for 15 min. before the antioxidant capacity evaluation). The antioxidant activity (percentage of scavenging of free radicals) of carotene – NLCs prepared with 0%, 2.5% and 5% trehalose was calculated by using the relation:

$$\%AA = \frac{I_0 - I_s}{I_0} \cdot 100$$

where: I_0 = the maximum CL for standard at t = 5s; I_s = the maximum CL for sample at t = 5s.

2.3.5. In vitro determination of SPF

The determination of SPF indexes was realized by using UV-VIS V670 Spectrophotometer equipped with integrated sphere and the adequate soft. For sun protection factor evaluation (SPF), an amount of 2 mg/cm^2 cream that contains the OCT-SLNs is applied onto a synthetic skin (Transpore™ 3M support) and the sample spectrum is registered on 290-400 nm, by using a reference support – Transpore™ 3M whitout cream. The method for *in vitro* determination of SPF is based on Diffey and Robson theory [22]:

$$SPF = \frac{\sum_{(400-290)} E_\lambda \cdot B_\lambda}{\sum_{(400-290)} \frac{E_\lambda \cdot B_\lambda}{MPF_\lambda}}$$

where: E_λ – sun radiation extinction for Earth (between 20° – 40° N latitude); B_λ – relative extinction for each wavelength; MPF_λ – the monochromatic protection factor for selected wavelength (the difference between the spectrum of measured sample applied on support and support spectrum).

2.3.6. UV-A and UV-B irradiation

The photostability of OCT-SLNs has been evaluated by irradiation on UVA-UVB domain with an energy of 19.5 J/cm^2, at two wavelengths: 365 nm (UVA) and 312 nm (UVB) on a short period (1h on UVA and 2h on UVB – *irradiation I*) and prolonged period of time (2h on UVA and 4h on UVB – *irradiation II*), using Irradiation System BioSun, Vilver Lourmat, France. The extent of photodegradation was monitored by recording the diffuse reflectance spectra in the wavelength range of 290-400 nm on a UV-VIS V670 Spectrophotometer (Jasco, Japan), by using the accessory with integrated sphere.

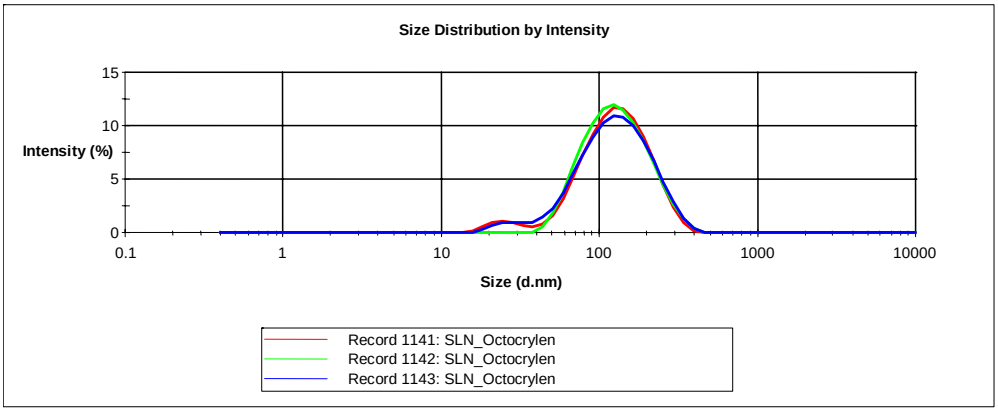
3. Results

Different biological lipids consisting in triglycerides, wax and some natural liquid oils (squalene and grape seed oils) were considered for production of free-SLNs and NLCs and loaded with the selected active compounds, by using a modified high shear homogenization technique coupled with the melting emulsification. The lipid formulations were produced using binary surfactant mixtures (ionic and non-ionic mixture) supplemented by a block copolimer as co-surfactant.

Characterization of SLNs and NLCs loaded with the selected active compounds by DLS

Starting from the idea that an adequate characterization of the initial lipid nanodispersions is a necessity for the control of the quality of the final desired product, the particle size parameters of aqueous dispersions of SLNs and NLCs have been evaluated by using dynamic light scattering technique, immediately after their production.

Generally, the size distribution of SLNs was larger than those of NLCs. The mean diameters evaluated by DLS technique varies from 100 to 230 nm in case of SLNs loaded with octocrylen and between 85 ÷ 139 nm, for NLCs loaded with β-carotene and prepared with various natural liquid oils. The size distribution for two optimized samples of SLN and NLC loaded with octocrylen and carotene, is represented in figure 2. The main diameter of lipid nanoparticles prepared in the Tween 80/Lecithin/ Synperonic/GS/CP system with 2.5% trehalose was 105.4 ± 0.519 nm (Pdl = 0.232 ± 0.009) (fig. 2a), while in the case of lipid nanocarriers prepared with Tween 20 as main surfactant and grape seed oil as natural liquid oil, a narrow size distribution was observed with a polydispersity index of 0.184 ± 0.004 and a mean diameter of 85.7 ± 2.136 nm (Fig. 2b).



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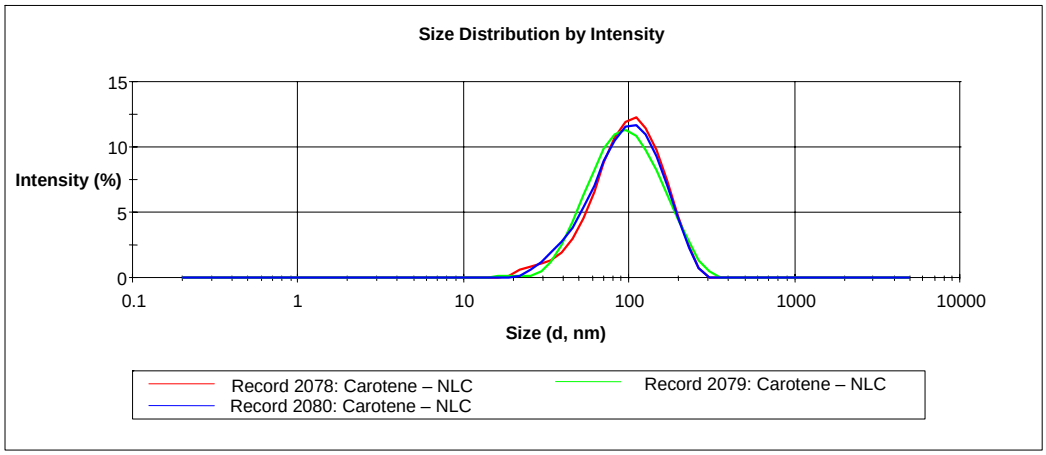

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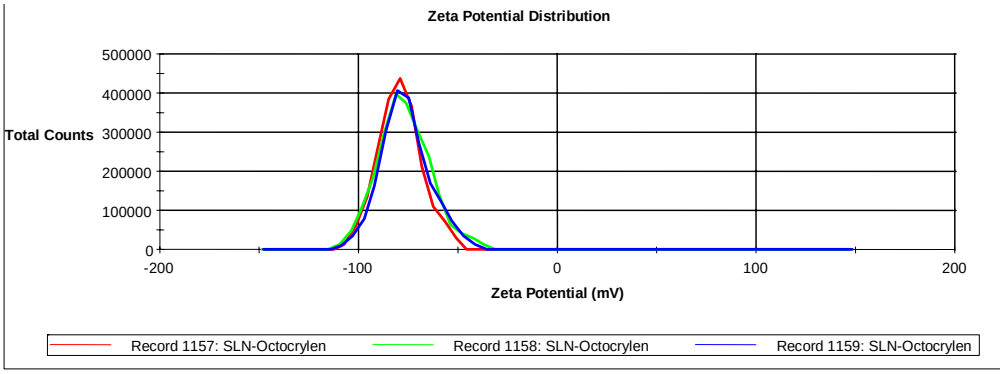
Fig. 2. Size distribution of lipid nanoparticles loaded with 1% octocrylen (a) and lipid nanocarriers loaded with 0.07% carotene (b), evaluated by DLS technique.

The physical stability of octocrylen – SLNs and carotene – NLCs

The key factor to evaluate the physical stability of colloidal dispersions is reflected by the electric charge on the particle surface, meaning the values of electrokinetic potential. The zeta potential (ZP) values obtained for the octocrylen-SLN and carotene-NLC aqueous suspensions meets the required conditions for a good stability in time under the investigated condition. All the prepared lipid nanoparticles showed a strong negative electrokinetic potential, with values between –68 mV ÷ –72 mV (fig 3a, for octocrylen-SLN prepared in the Tween 80/Lecithin/ Synperonic/GS/CP, with 2.5% Trehalose), and –29 mV ÷ – 41 mV (fig. 3b for carotene-NLC prepared in the same system, but with Tween 20 as main surfactant and grape seed oil as natural lipid). The zeta potential distribution for an octocrylen-SLN and a carotene-NLC system is exemplified in figure 3. The most stable system has been that prepared with Tween 80 as main surfactant, 1% octocrylen and 2.5% trehalose, with a mean potential value of - 72.1 ± 1.86 mV (fig. 3b). These values demonstrated that the loaded – SLNs and NLCs obtained by a modified-high shear homogenization method are physically stable systems, they presenting a high tendency to reject particles and therefore there is a less likely aggregation of lipid nanoparticles in aqueous dispersion.



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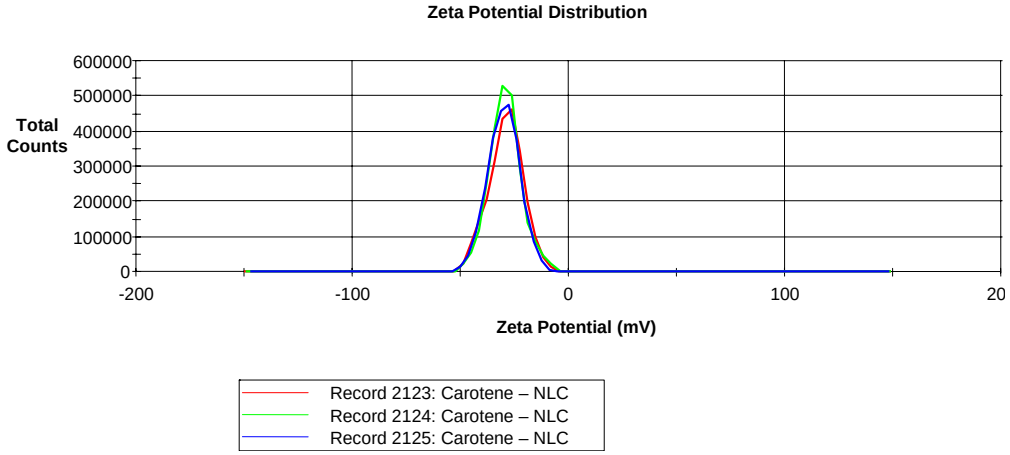

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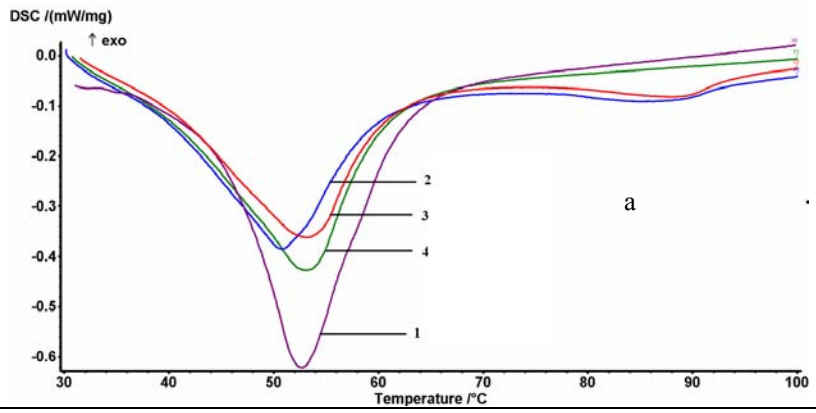
Fig. 3. The zeta potential distribution of: a. octocrylen – SLNs; b. carotene – NLCs

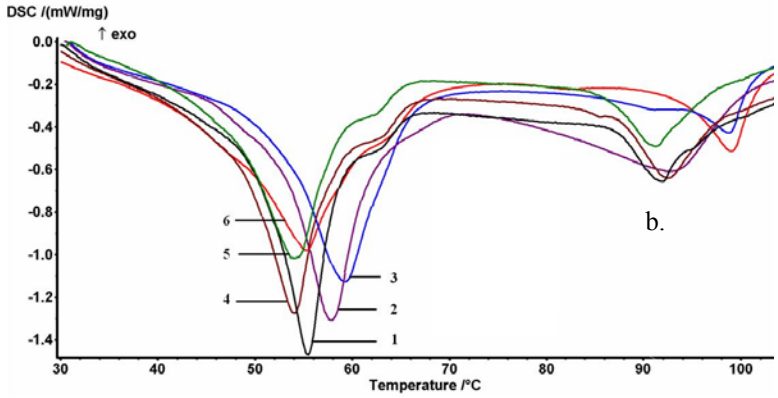
The structural characterization of free- and loaded – lipid nanoparticles by differential scanning calorimetry

The lipid crystalline structure by means the solid state of the particles and types of polymorphs that can be formed, determines the entire benefits of SLN and NLC systems. In these systems, it has been demonstrated that amorphous lipid structures provide superior drug loading and retention than more crystalline structures [23]. As the lipid nanoparticles transitioned from less ordered to more ordered solid states, the nanoparticles exibit drug release.

The evaluation of crystalline structure of octocrylen – SLNs and carotene – NLCs achieved by differential scanning calorimetry revealed that: (1) by a heating cycle from 30 to 100° C, two main endothermic peaks were observed, one specific for the heterogeneous lipid mixture (50-60^{o}C) and another corresponding to the cryoprotector melting (85-100^{o}C); (2) all the loaded – nanoparticles present a less ordered crystalline arrangement of lipid matrices as comparing with those of free-lipid nanoparticles; (3) the repartition of cryoprotectors depends on the nature of lipid matrix and carotene concentrations. The calorimetric peaks for the free- and loaded-SLNs and NLCs prepared with Tween 20 or 80 as main surfactant are presented in figure 4 a and b.



Legend
1, 4 = Free – SLN and loaded with 1%OCT, without Trehalose
2, 3 = SLN loaded with 1% and 2% OCT, respectively, prepared in Tween 80/Lecithin/Synperonic/GS/CP system, with 2.5% Trehalose



Legend
1, 2, 3 = Free-NLC prepared in Tween 20/Lecithin/Synperonic/ GS/CP and **GSO** system with 2.5% Trehalose and loaded-NLC with 0.05% carotene and 0.07% carotene, respectively.
4, 5, 6 = Free-NLC prepared in Tween 20/Lecithin/Synperonic/ GS/CP and **SQ** system with 2.5% Trehalose and loaded-NLC with 0.05% carotene and 0.07% carotene, respectively.

Fig. 4. DSC curves for: a. octocrylen – SLNs; b. carotene – NLCs

The in vitro evaluation of antioxidant and anti-UV properties of octocrylen – SLNs and carotene – NLCs prepared with and whithout cryoprotectors

For the study of cryoprotective agents influence, a large domain of mannitol concentrations (between 2.5-15%) have been used in order to establish if there are some significant effects on the anti-UV properties of SLN loaded with octocrylen. By determining the anti-UV properties, a concentration over 5% mannitol has lead to a gradually decreasing of SPF values (fig. 5), while 2.5% trehalose have demonstrated an increasing of SPF index (fig. 6). Based on these results, only 2.5 and 5% cryoprotector concentrations have been further used for observing the antioxidant behaviour of NLCs loaded with carotene. By evaluating the ability of carotene – lipid nanocarriers to scavenge free radicals, it has been shown that the presence of trehalose produced a significant effect on the antioxidant behaviour of nanoparticles, meaning a decreasing of specific property with increasing of cryoprotective concentration (fig. 7, 8), for both kinds of natural liquid oil (squalene or grape seed oil) used.

4. Discussion

The presence of the two natural oils (squalene and grape seed oil) in the lipid composition has leads to a decrease of particle size, as comparing with those of SLNs systems (fig. 3). The lower size encountered in case of NLCs can be explained by an optimized crystalline arrangement of lipid particles, as may be observed from the calorimetric curves (fig. 4). NLCs present narrower and sharper calorimetric peaks than SLNs. Moreover, by observing the peak allure in the region of 85-100°C, there is a shift of trehalose melting temperatures in case of the two SLN and NLC systems, completed by an evident repartition of cryoprotector as a function of lipid matrix type. Only a flatted shoulder appeared in case of SLNs at about 86°C (fig. 4a), which confirms the presence of trehalose outside the lipid core (in the outer surfactant shell), most probably due to a weak association with the hydroxyl groups from polyoxyethylenesorbitan surfactant. In case of NLCs the trehalose melting is well localized at about 92.1 °C and 98.8 °C (fig. 4b). This last aspect could

be associated to a repartition of trehaloze inside the lipid network.

In terms of anti-UV-properties of SLNs prepared with various mannitol and trehalose concentrations, for some cosmetic formulations that contain 1.5% (fig. 5) and 2.5% (fig. 6) octocrylen, it has been evaluated the SPF index. From figure 5 it has been shown that a concentration of 2.5% mannitol has lead to an increase of SPF, while a concentration over 5% mannitol induces a decrease of it. The presence of a minimum concentration of mannitol enables the formation of hydrogen bonds that allow an efficient energy dissipation, manifested as a broadening of vibronic absorbtion domain. At a higher mannitol concentration (> 10%) the sunscreen effect of octocrylen has gradually inhibited (Fig.5).

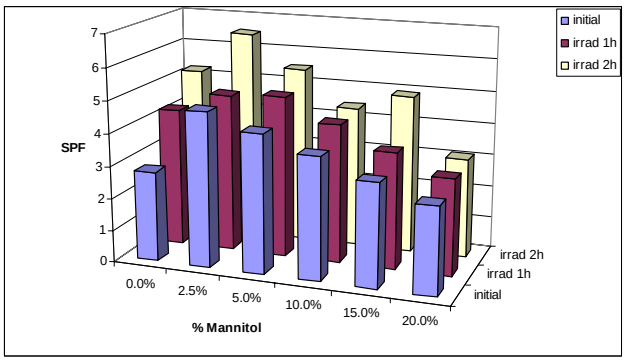


Fig. 5. Influence of manitol concentrations on the SPF index, before and after controlled irradiation.

This presumption is also sustained by the photostability studies achieved in mild irradiation conditions (fig. 6). After photoexcitation, the hydrogen bonds enable an excited state proton transfer (photo-tautomerism), which results in a rapid conversion of UV light to vibrational energy. This explains the improving of SPF index encountered when using mannitol or trehalose (fig. 6), after both controlled irradiation stages.

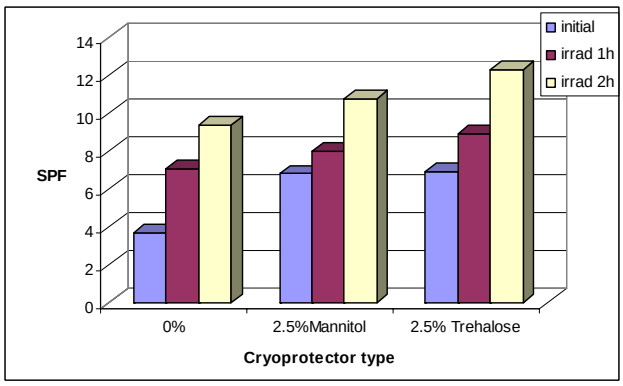
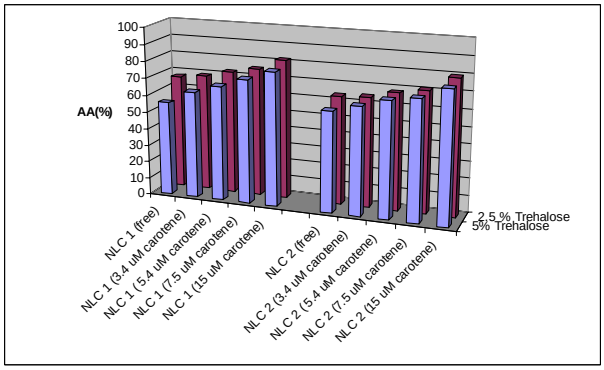


Fig. 6. Effect of cryoprotector type on the anti-UV properties, before and after controlled irradiation (2.5% octocrylen).

Regarding the antioxidant properties, in some studies [24, 25] it has been found that trehalose acts as antioxidant. Despite these aspects, in this study, the trehalose decreased the antioxidant activity manifested by the cumulated effect of carotene with natural antioxidant oils (squalene or grape seed oils). The decreasing of AA% with increasing of trehalose concentration is mainly attributed to the mild alkaline environment used in the chemiluminescence generator system for creating the free radicals and also could be influenced by the using of ethanol (with pro-oxidant effect).



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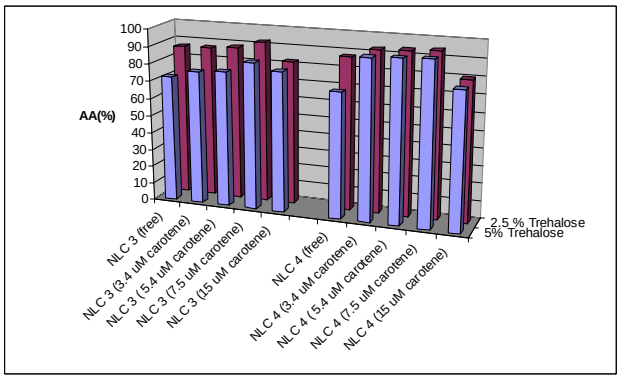

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Fig. 7. The variation of antioxidant activity of carotene-NLCs prepared with Sq (a) or GSO (b), as function of carotene and trehalose concentrations. NLC 1 and 3 are prepared with Tween 20 as main surfactant, while NLC 2 and 4 are prepered with Tween 80.

The alkaline media favors the dissociation of hydroxyl groups of trehalose and thus the easy formation of some additional oxygenated free radicals, beside those originated from the luminol/H_2O_2 system.

A representative exemple that underlines the decreasing of antioxidant capacity in presence of trehalose has achieved on samples prepared with a concentration of 15μM carotene (fig. 8). From figure 8 it can be observed a significant influence of trehalose on the antioxidant activity, mainly manifested in the NLC prepared with grape seed oil and Tween 20/Lecithin/Poloxamer system.

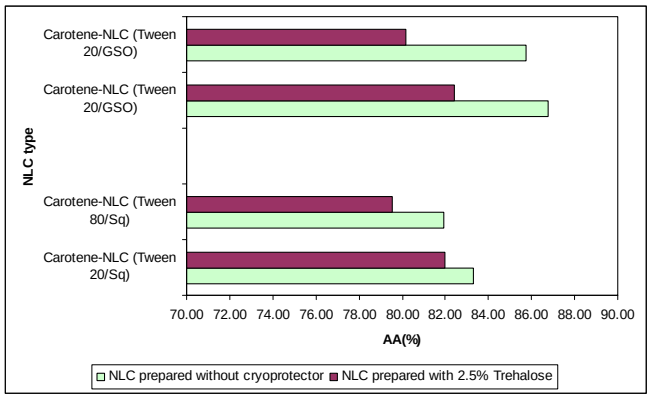

Fig. 8. Effect of trehalose on the antioxidant activity of a sample of carotene – NLC.

5. Conclusion

The behaviour of two types of cryoprotectors, on the antioxidant and anti-UV properties of octocrylen – lipid nanoparticles and carotene – lipid nanocarriers have been analysed in this study. Both kinds of cryoprotective agents have shown to have a significant influence on the main properties of developed loaded – SLNs and NLCs.

The presence of mannitol and trehalose has leads to an increase of SPF index, manifested for both, initial and irradiated cosmetic formulations enriched with lipid nanoparticles. The increasing of the SPF has been assigned to the formation of hydrogen bonds that allow a rapid conversion of UV light to vibrational energy. These kinds of physical bonds have as result a broadening effect of vibronic absorbtion domain.

The use of trehalose decreased the antioxidant activity of carotene – NLCs. The appearance of a pro-oxidant effect could be mainly associated to presence of a mild alkaline environment that favors the dissociation of hydroxyl groups of trehalose and thus the easy formation of some additional oxygenated free radicals.

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References

[1] R.H. Müller, M. Karsten, G. Sven, Eur J. Pharm. Biopharm. **50,** 161 (2000).
[2] K. Westesen, "Particles with modified physico-chemical properties, their preparation and uses, US Patent No. 6197349, (2000).
[3] M.E. Barbinta Patrascu, A. Cojocaru, L. Tugulea, N.M. Badea, I. Lacatusu, A. Meghea, J. Optoelectron. Adv. Mater. **13**, 1165 (2011).
[4] M.E. Bărbînță-Pătrașcu, L. Țugulea, Rom. Journ. Phys. **50**(9), 1171 (2005).
[5] T. Zhang, J. Chen, Y. Zhang, Qi Shen, W. Pan, European J. of Pharm. Sci. **43,** 174 (2011).
[6] J.Y. Fang, C.L. Fang, C.H. Liu, Y.H. Su, Pharmaceutics and Biopharmaceutics 70, 633 (2008)
[7] A. Hentschel, S. Gramdorf, R.H. Muller, T. Kurz. Nanoscale Food Science, Eng. and Technol. **73,** 1, (2008).
[8] L.R. Gaspar, PMB Campos, Int. J. Pharmaceutics **343,** 181 (2007).
[9] A. del Pozo-Rodríguez, M.A. Solinís, A.R. Gascón, J.L. Pedraz, European J. of Pharm. and Biopharm. **71,** 181, (2008).
[10] W. Mehnert, K. Mäder, Adv. Drug. Deliv. Rev. **47,** 165 (2001).
[11] J. Pardeike, A. Hommoss, R.H. Müller, Int. J. Pharm. **366,** 170 (2009).
[12] S.A. Wissing, R.H. Müller, Int. J. of Pharmaceutics, **254,** 65 (2003).
[13] S. González, M. Fernández-Lorente, Y. Gilaberte-Calzada, Clinics in Dermatology **26,** 614 (2008).
[14] M.V.R. Velasco, F.D. Sarruf, I.M.N. Salgado-Santos, C.A. Haroutiounian-Filho, T.M. Kaneko, A.R. Baby, Int. J. of Pharm. **363,** 50 (2008).
[15] D.L. Giokas, A. Salvador, A. Chisvert, Trends in Analytical Chemistry, **26**(5), 345 (2007)
[16] S.A.Wissing, R.H. Muller, Pharmazie **56**, 783 (2001).
[17] M. Demirel, Y. Yazan, RH Müller, et al. J. of Microencapsul. **18,** 359 (2001).
[18] T. Helgason, T. S. Awad, K. Kristbergsson, D.J. Mcclements, J. Weiss, J. Colloid and Interface Science, **334,** 75 (2009).
[19] K. Jores, W. Mehnert, M. Dreschsler, H. Bunjes, C. Johann, K. Mader, J. Controll. Realease **95,** 217 (2007).
[20] S. Trombino, R. Cassano, R. Muzzalupo, A. Pingitore, E. Cione, N. Picci, Colloid and Surfaces B: Biointerfaces **72,** 181 (2009).
[21] I. Lacatusu, M.N. Badea, A. Murariu, A. Meghea, Nanoscale Research Letters 6, 73 (2011).
[22] B.L. Diffey, Robson, R., J. Soc. Cosmet. Chem, **40**, 127 (1989).
[23] M.D. Triplett, J.F. Rathman, J. Nanopart. Res. **11**, 601 (2009).
[24] N. Benaroudj, D.H. Lee, A.L. Goldberg, J. of Biological Chem. **276**(26), 242 (2001).
[25] Y. Luo**,** W.M. Li**,** W. Wang, Environmental and Experimental Botany, **63**(1-3), 378 (2008).

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