NIOSOMES LOADED WITH ASCORBIC ACID – INFLUENCE OF SURFACTANTS ON STABILITY AND MORPHOLOGY

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The purpose of this study was to develop niosomes, nanostructured systems capable to incorporate and maintain hydrophilic active principles, of interest in the cosmetics industry. The selected preparation method was film hydration, influence of HLB index of the surfactant, the effect of surfactant : cholesterol ratio and others process related parameters were studied in order to obtain niosomal vesicles with desired properties and stability. Morphology, dimensions and stability were assessed by optical microscopy, Dynamic Light Scattering (DLS) and zeta potential measurements.

Keywords: niosome, surfactant, cholesterol, active principles, cosmetics industry, ascorbic acid

1. Introduction

"No one dies of an old skin; however worn the covering after the battering of a lifetime, it still performs its original protective function" - Albert Kligman"

Skin aging is a complex biological process, a result of the interaction of genetic, intrinsic and extrinsic factors and not yet elucidated. People around the world searched "anti-aging" so the cosmetic industry developed in 80s., and the companies try to find actives ingredients that can help to counteract the perceptible effects of aging [1].

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In recent years nanostructures have been extensively used in almost all areas of activity. The use of nanotechnology, more precisely transport and release of active compounds, is evolving in the medical and pharmaceutical industries. Niosomes were first discovered by Handjanivila and his collaborators in 1979 [2]. They are stable with good penetration power and less irritant compared to other colloidal carriers [3].

Niosomes are non-ionic surfactant vesicles, similar to liposomes, but are preferred over liposomes due to several factors: liposomes' double layer membrane needs phospholipids which are more sensitives to oxidation, becoming instable, while niosomes do not need in their composition phospholipids, they are formed by non-ionic surfactants and choresterol; to maintain the stability of the liposomes, storage in an inert atmosphere of nitrogen is required; phospholipids used in the preparation of liposomes have natural origin so their purity is variable and often involves additional purification. All these problems contribute to a higher price in the formulation of liposomes and taking in consideration the above mentioned advantages of niosomes, their preparation is less expensive and the resulted vehicles are more stable as compared with liposomes. Moreover, niosomes offer a lot of advantages in cosmetic products due to their capacity to penetrate the skin. These vesicles based on non-ionic surfactants and cholesterol , a constituent of skin lipids, increase the permeability of the encapsulated substances through the skin, ensuring efficient delivery and release of the cosmetic active ingredients to the target. Niosomes are biodegradable, biocompatible and non-toxic, having a low production cost and being easy to handle and store [5, 6].

Niosomes formation requires the presence of a particular class of amphiphilics and hydration solvent and also additives to stabilize the system against the formation of aggregates. Other factors that contribute to the formation of niosomes are the value of HLB (Hydrophilic – Lypophilic Balance) of the amphiphilic molecule, the hydration medium, the length of the lipid chain, the assembling and the symmetry of the membranes [7-8].

Until May 2017, approximately 5400 researches with "anti-aging" subject have been reported in Science and Technology field. In these reports more than 300 compounds with anti-aging activity have been mentioned [4], vitamin C being one of the most important anti-aging active since 1980s.

Ascorbic acid or vitamin C presents an important antioxidant activity and maintains the vitamin E in his active form in the organism. Also it plays an important role in collagen synthesis, thus reducing the appearance of fine lines and wrinkles, improves skin tone for a healthier look and neutralizes free radicals against photo-aging. Vitamin C is a white or slightly yellow powder, odorless, with acid character and hydrophilicity (Fig.1). The use as cosmetic active is impaired by its poor stability, being easily degraded in oxidation pathways, as

well as by the difficulty of penetrating into the skin due to the skin structure and its hydrophilicity. A possibility to overcome these issues is to use a delivery system, for example niosomes.[2-3]

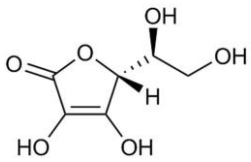


Fig. 1. The chemical structure of the Ascorbic Acid

As mentioned above, advantages of niosomes are determined by the two major components used in preparation of these systems, non-ionic surfactants and cholesterol [7].

Cholesterol

Cholesterol is used in niosomes to ensure the rigidity and the shape of the vesicle due to its interactions with the non-ionic surfactant, increasing the stability and the entrapment capacity of the vehicle. Cholesterol, an amphiphilic molecule (Fig. 2), interacts with surfactants by hydrogen bonding between the hydroxyl group and polar moieties of the surfactant as well as by van der Waals bonds between its side chain and hydrophobic chain of the surfactant, leading to increased mechanical stiffness and cohesion of the double layer membrane. Cholesterol also has a condensation effect as it is found in the cavity formed by surfactant monomers. This is known as the cholesterol-filling function, due to which the carbon - carbon movement is restricted, which leads to a decrease in the permeability of cholesterol containing membranes compared to cholesterol free membranes. The amount of cholesterol used in preparation of niosomes depends on the HLB value of the surfactants. By increasing the HLB value (above 10), the hydrophilic moiety of the surfactant is larger and supplementary quantity of hydrophobic cholesterol is required [9-11].

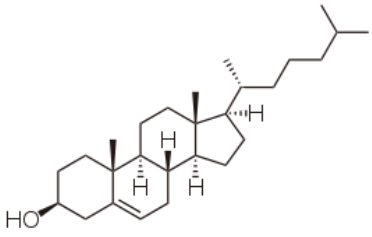


Fig. 2 The chemical structure of the Cholesterol

Non-ionic surfactants

The main components of niosomes are represented by non-ionic surfactants with established benefits such as superior stability, diminished toxicity and increased compatibility as compared to anionic, amphoteric or cationic analogs. Non-ionic surfactants which are commonly used in niosome preparation are alkyl esters and ethers, sorbitan fatty acids esters or polyoxyethylene sorbitan fatty esters or sucrose fatty acid esters possessing a hydrophobic moiety consisting of 1-2 hydrocarbonated chains with lengths between 12 and 18 carbon atoms. The formation of a bilayer vesicle depends on the hydrophilic–lipophilic balance (HLB) of the surfactant which influences also the entrapment capacity, hydrophilic actives encapsulation being favorized by high values of HLB. The entrapment efficiency depends also on the hydrocarbonated chain length, surfactants with longer chains such as stearyl (C18) producing more entrapment efficient niosomes than the lauryl (C12) analogs [5, 12, 30]

2. Materials and methods

Materials:

Polyoxyethylene (20) sorbitan monooleate, Tween 80 (Fisher BioReagents), Sorbitane monooleate**,** Span 80 (Merck), Cholesterol 95% (Across Organics), Chloroform (Sigma-Aldrich), Vitamin C (Merck) were of analytical grade and used with no further purification. Ultrapure water (18.2 MΩ) was used for all experiments. Extrusion of the niosomes was performed using PES filters with diameter pores of 0,22 μm.

Methods:

Preparation of Niosomes: niosomes were prepared by thin film hydration method using two surfactants (Tween 80 and Span 80) with cholesterol at 5:1 and 4:2 ratio (*Table 1*) [13]. In a typical experiment a mixture of surfactant and cholesterol in the selected ratio was dissolved in 5 mL chloroform. The film was obtained by vacuum evaporation using a rotavapor at 35˚C, for 30 minutes at a speed of 100 rpm. For some experiments the obtained film was maturated for 24 h at 4^{o}C [13, 17].

Film hydration was performed by addition of 5 mL of Vitamin C 1% solution and homogenized following two protocols:

- A. Sonication for 30 min or 60 min at 60^{o}C;
- B. Homogenization 30 min at 60^{o}C at 90 rpm using the rotavapor;

The obtained niosomal dispersion was transferred into a bottle glass, for the further analyses.

Ultrasound treatment of niosomal dispersions was performed with Sonorex Digitec, Bandelin sonicator

The morphology of hydrated niosomal dispersion was examined by optical microscopy using an optical camera microscope (METTLER FP52) at x10 and x40 magnification [16-18]. A drop of the niosomal dispersion was placed on microscope blade and subjected to a microscopic analysis to observe the shape of the vesicle, photographs being taken at x10 and x40 magnification.

The particle size parameters of niosomes given by the hydrodynamic diameters $Z_{average}$ and polydispersity index, PDI of each niosomes dispersion were determined by dynamic light scattering (DLS) technique, using a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom), equipped with a solid-state laser (670 nm). Aqueous dispersions of the obtained niosomes were measured at a 90^o scattering angle at 25^{o}C. The particle size data were evaluated using intensity distribution. The average diameters (based on Stokes–Einstein equation) and the PDI were given as average of three individual measurements Dispersions were analyzed after appropriate dilution with deionized water to an adequate scattering intensity prior to the measurement. The particle size analysis data were evaluated using intensity distribution [19-21].

Zeta potential (ZP) was determined by measuring the electrophoretic mobility of nanoparticles in an electric field with the appropriate accessory of the Zetasizer Nano ZS. ZP reflects the electrical charge of the particle surface and is a measure of the physical stability of the colloidal system [15, 22].

UV-VIS Spectrophotometry was performed on a Thermo Scientific Evolution 220 UV-Visible Spectrophotometer, with 1.0 nm Resolution and Double-beam Configuration.

Entrapment efficiency of ascorbic acid was determined by UV spectroscopy according to a method reported in the literature [14]. Thus a 2 mL niosomes sample dispersion was inserted in cellulose dialyzing membrane (12000 Da). The solution was dialyzed versus a 200mL volume of ultrapure water for 15 min and the untrapped ascorbic acid concentration was determined by UV spectroscopy at 263.1 nm, using a calibration curve.

Entrapment efficiency was calculated according to the formula:

$$EE\% = Cp / Ct \times 100 \quad (1)$$

Where Cp represents the concentration of encapsulated ascorbic acid and Ct is the initial ascorbic acid concentration.

3. Results and discussion

Niosomes containing vitamin C were prepared using two type of non-ionic surfactants (Span 80 and Tween 80) and cholesterol in different proportions and a fixed concentration of vitamin C aqueous solution, by lipid film hydration technique. The selection of Tween 80 and Span 80 surfactants with common

feature the existence of an oleyl hydrophobic moiety for the preparation of niosomes was made according to the reported literature data stating that these surfactants lead to the formation of stable niosomes [23-24] which may successfully encapsulate different actives such as curcumin [14] and capsaicin [28]. According to reported studies non-ionic surfactants of 4-8 HLB value are compatible with vesicle formation, so Span 80 with HLB value of 4.3 is included in this range [23]. The more hydrophilic surfactant Tween 80 (HLB value 15) may favor an increased entrapment efficiency for the employed hydrophilic active, ascorbic acid. Its use in niosome preparation is reported to favor formation of stable vehicles with applications in pharmaceutical field [14, 25]. The molar ratio of non-ionic surfactant:cholesterol was selected according to reported data in order to ensure not only a suitable hydrodynamic diameter, shape and rigidity of the vesicle but also satisfactory entrapment efficiency for the obtained niosomes [9-11]. The selection of film hydration method for the manufacturing process was made because it is faster, easier and simpler to use.

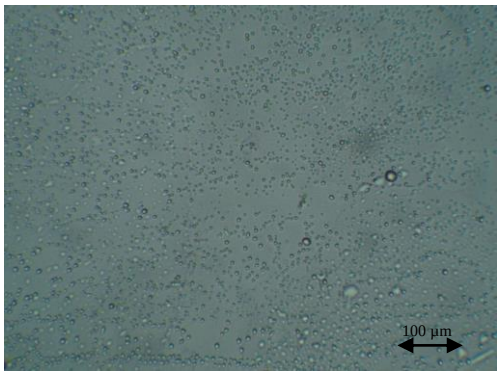
Table 1

Molar ratio of components and synthetic routes for the prepared niosomes

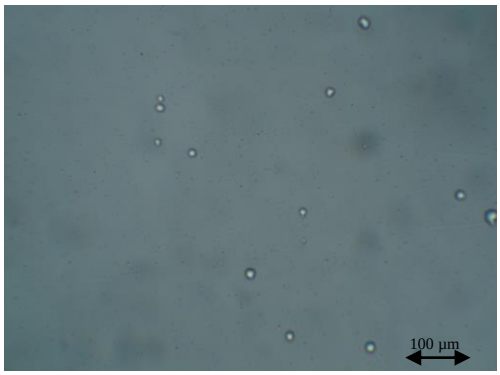
Formulation code	Span 80/ Cholesterol	Tween 80 / Cholesterol	Maturation time (h)	Sonication
	Molar ratio	Molar ratio		Time (min)
F1	5:1	-	24	15
F2	5:1	-	24	30
F3	5:1	-	24	60/60
F4	-	5:1	24	60
F5	4:2	-	24	60
F6	-	4:2	24	60
F7	5:1	-	0	30
F8	5:1	-	24	30
F9	-	5:1	24	30
F10	-	5:1	0	30

The influence of sonication time on formation and stability of niosomes during rehydration of the obtained lipid film was achieved by performing three rehydration experiments of the lipid film obtained from Span 80 and cholesterol at 5:1 ratio with sonication time of 15, 30 and 60 min respectively (**F1-F3**).

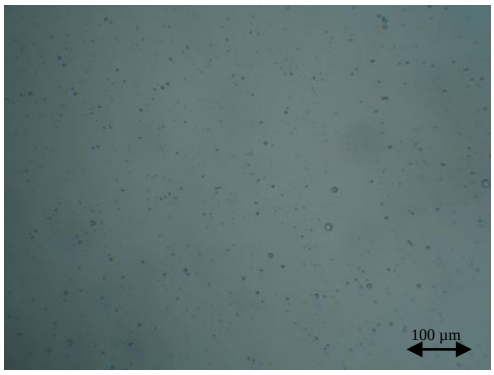
The morphology of the prepared vesicles was assessed using optical microscopy under x10 and x40 magnification, respectively (Fig. 3). Spherical vesicles with smooth surface and with a diameter below 5 - 10 μm were obtained for the **F1 – F3** formulations prepared with Span 80: cholesterol in 4:1 ratio (Fig.3 a-c). Increasing of the sonication time at 60 min resulted in reduction of the niosomes size (30-50 %.) but accompanied with significant destruction of the vesicles (Fig 3- c).



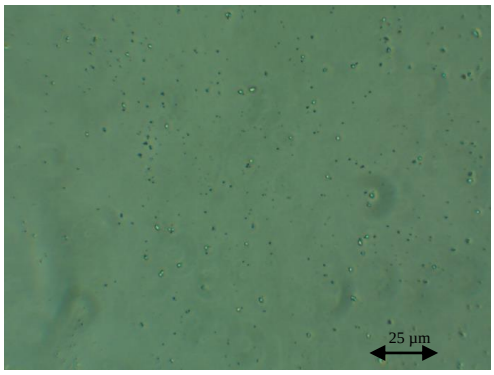
a) Niosomes (Span 80) - 15 min of sonication (**F1**)



b). Niosomes (Span 80) -30 min of sonication (**F2**)

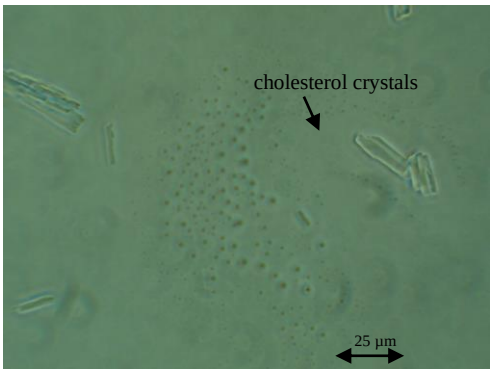


c). Niosomes (Span 80) - 60 min of sonication (**F3**)



d) Niosomes (Tween 80) 60 min of sonication (**F4**)

e). Niosomes with Span 80 : cholesterol (4:2)(F5)



f) Niosomes with Tween 80 : cholesterol (4:2) (F6)

Fig. 3 Optical microscopy images for the formulated niosomes **F1-F6**

Formulation of niosomes with Tween 80 :cholesterol in the same molar ratio of 4:1 with 60 minutes of sonication (**F4**) resulted in 50% smaller vesicles (Fig. 3- d) suggesting that the nature of surfactant influenced the size of the vesicle in good agreement with literature data [26]. In general, the increased amount of cholesterol in niosomes formulations is supposed to yield more stable vesicles especially in the case of hydrophilic surfactants such as Tween 80. For

Span 80 and Tween 80 prepared formulations (**F5** and **F6** respectively) with increased ratio of cholesterol (4:2) optical micrographs revealed the formation of cholesterol crystals (Fig. 3- e, f) so these experiments were abandoned.

In conclusion the preparation parameters were chosen as follows: surfactant: cholesterol molar ratio of 5:1 and sonication time at rehydration of the lipid film 30 min.

These parameters were respected for preparation of formulations **F7- F10** for which the influence of the lipid film maturation time was also investigated. An addition extrusion process after rehydration and sonication was added to the procedure using PES filters with a pore diameter of 0.22 μm in order to reduce the formation of multilamellar vesicles. The particle size, polydispersity index and stability of the obtained formulations was assessed by DLS. (Table 2). The lipid film obtained both from Span 80 and Tween 80, hydrated immediately after evaporation step and subsequently extruded yielded vesicles with low negative value of zeta potential but with a high polydispersity index, especially for Span 80 formulation **F7**. Maturation of the lipid film for 24 hrs did not significatively influence the hydrodynamic diameter of the obtained niosomes but decreased the polydispersity index in the case of Span 80 formulation (**F8**) with 40%. The mean diameter determined by DLS (Table 2) is in good agreement with the optical microscopy observations, Tween 80 niosomes (**F9** and **F10**) are 50% smaller than niosomes obtained by using Span 80 (**F7** and **F8**).

Table 2

Particle size, polydispersity index and stability of niosomal formulations F7-F10

Formulation code	Maturation time (h)	DLS		
		Particle size (μm)	PDI	Zetapotential (mV)
F7	0	0.223 ± 0.066	0.702 ± 0.207	-7.06 ± 0.804
F8	24	0.262 ± 0.012	0.425 ± 0.013	+ 0.501 ± 0.313
F9	24	0.111 ± 0.019	0.293 ± 0.224	+ 0.395 ± 0.212
F10	0	0.112 ± 0.009	0.272 ± 0.013	-6.06 ± 1.04

For the niosomes obtained by hydration of a 24 hrs maturated lipid film (**F8**, **F9**) the values of zeta potential are positive and even lower than the values obtained after hydration of unmaturated film probably due to segregation of lipid components during maturation process resulting in an unstable membrane.

All the analyzed niosomes formulations have modest values for the zeta potential indicating a low stability. Another explanation may be given by the nature of Span 80 (Fig.4) and Tween 80 (Fig.5) which contain oleic acid moieties

with a *cis* double bond in the middle of the chain causing a bending of the chain and affecting the value of the critical packing parameter (CPP), an important factor for formation and stability of vesicles. Thus, the formed bilayer membrane is not so tightly packed presenting a certain degree of permeability, previously reported in the literature when similar polar head surfactants but with different hydrophobic moieties Tween 60 (stearic acid) and Tween 80 (oleic acid) were involved in the preparation of niosomes and their permeability have been compared [31].

Literature data suggest that increased values of zeta potential may be achieved when charged additives are used in synthesis of niosomes such as negatively charged dicetyl phosphate (DCP), sodium lauryl sulfate (SLS) or positively charged stearylamine (SA) and cetylpyridinium chloride in 2.5- 5mol% ratio. [10]

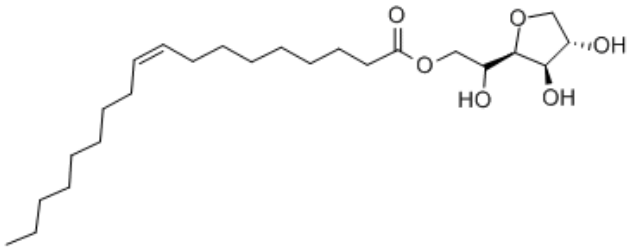


Fig. 5. Span 80 Structure

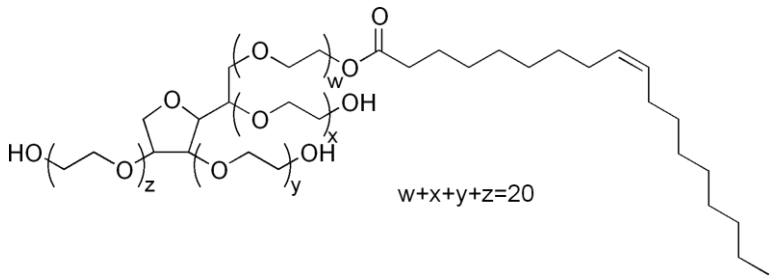


Fig. 6. Tween 80 Structure

Modest entrapment efficiency values of ascorbic acid were determined for all the examined formulations **F7-F10** as presented in Table 3.

Table 3

Entrapment efficiencies of ascorbic acid in niosomes formulations F7-F10

Formulation code	Span 80: Cholesterol Molar ratio	Tween 80: Cholesterol Molar ratio	Drug entrapment efficiency (%)
F7	5:1	-	54.63±1.21
F8	5:1	-	53.24±0.87
F9	-	5:1	35.72±1.15
F10	-	5:1	34.20±0.95

Ascorbic acid was more successfully encapsulated in Span 80 formulations (**F7-F8**) with 53-54% entrapment efficiency in good agreement with data obtained for reported niosomes using hydrophobic Span type surfactants [28].

Although the use of a more hydrophilic surfactant could favor the encapsulation of ascorbic acid, the entrapment efficiency for Tween 80 niosome formulations decreased with 35% as compared to Span 80 counterparts. The obtained values are consistent with reported data for antioxidants encapsulated in Tween based niosomes [29]. The higher hydrophilicity of Tween 80 may favor retention of ascorbic acid on the surface of the vesicle rather than encapsulation inside of it, and this amount of active is removed in the experimental conditions for entrapment efficiency determination (dialysis versus water) increasing the amount of unencapsulated drug and thus lowering the value of entrapment efficiency.

4. Conclusions

In this study we have obtained niosomes based on Span 80 and Tween 80 non-polar surfactants with different surfactant : cholesterol ratio used for the encapsulation of a cosmetic active, ascorbic acid. The increase amount of cholesterol leads to partial crystallization of it, suggesting that these formulations are not stable. It was demonstrated that the niosomes size depends on de nature of the surfactant, Tween 80 leading to smaller vesicles. An increased time for sonication treatment from 15 to 30 min in the film hydration step decrease the size of niosomes but extended to more than 60 min. may damage the vesicles. Maturation of the lipid film prior to hydration or extrusion to PES membrane influence the size and stability of the obtained niosomes. Entrapment efficiency for ascorbic acid in the prepared niosomes had modest values between 30-50 % , the better results being obtained for Span 80 formulations.

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