Universidade de Lisboa

Faculdade de Farmácia

Development and characterization of niosomes as new drugs delivery systems

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Mestrado Integrado em Ciências Farmacêuticas

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

A utilização de niossomas para a veiculação de medicamentos, tem recebido muita atenção devido à potencial ação selectiva destas nanovesículas. Os niossomas são nanovesículas que se formam de forma espontânea, através da hidratação de tensioativos sintéticos. As propriedades dos niossomas são determinadas pelo seu tamanho, forma e pelas propriedades físico-químicas da sua superfície, sendo capazes de modificar a biodistribuição do medicamento e consequentemente a farmacocinética do fármaco por ele veículado.

Este trabalho começa por rever a literatura atualmente disponível sobre estas nanovesículas, relativamente aos diferentes componentes que são utilizados no seu desenvolvimento, métodos de preparação, fatores que afetam a sua formação, bem como metodologias utilizadas para a sua caracterização e principais aplicações.

O presente trabalho de investigação está integrado num projecto que tem como objectivo o desenvolvimento e caracterização de niossomas funcionalizados com receptores de manose, para a veiculação de fármacos anti-tumorais.

O trabalho realizado corresponde apenas a um dos primeiros objetivos deste projecto, o qual se focou no desenvolvimento e caracterização de niosomas vazios, tendo em vista a avaliação do impacto dos diferentes métodos de produção, bem como das diferentes proporções dos componentes da formulação nas propiedades físico-químicas destes sistemas. Foram testados três métodos diferentes para a produção de niossomas (TLE-Paddle, TLE-Vortex e Reverse Phase) e 6 proporções dos componentes dos niossomas (Span®60, colesterol e Solulan).

O tamanho, a dispersão de tamanhos (PDI), e o potencial dos niossomas foram determinados de forma a averiguar o impato das variáveis acima referidas. Os resultados apresentados permitem concluir que apenas o diferente rácio molar da formulação testada teve impacto nas características dos niossomas, uma vez que as propriedades dos niossomas não foram afetadas pelos métodos de produção. De entre as formulações de niossomas testadas, a proporção 50:25:25 de Span®60:Colesterol:Solulan permitiu produzir niossomas com as características desejadas, relativamente ao tamanho (inferior a 200 nm) e PDI (inferior a 0,2), sendo seguido pelo sistema produzido utilizando Span®60:Colesterol:Solulan na proporção 40:35:25.

Futuramente, os niossomas serão funcionalizados com o recetor de manose à superfície, será selecionado do fármaco antitumoral a veicular, far-se-á a preparação, produção e caracterização destes sistemas, bem como a avaliação da sua ação biológica.

Os niossomas constituem sistemas promissores para a veiculação direcionada de substâncias ativas. No entanto, há algumas limitações associadas à sua utilização, nomeadamente a sua translação para uso clínico e o custo de produção.

Palavras-Chave: Niossomas, Método de produção, Caracterização de niosomas

Abstract

The use of niosomes for the delivery of medicines has received much attention due to the potential selective action of these nanovesicles. Niosomes are self-assembled vesicular nanocarriers obtained by hydration of synthetic surfactants. The properties of niosomes are determined by their size, shape and physico-chemical characteristics of their surface, being able to modify the drug's biodistribution and consequently the pharmacokinetics of the entrapped drug.

As a background of the present work, a literature review was made on niosomes: their formulation components, methods of preparation, factors affecting their formation, physicochemical characterization and major applications.

This research work is part of a project that aims to develop and characterize functionalized niosomes for mannose-targeted antitumoral drug delivery. The work carried out corresponds only to one of the first objectives of this project: development and characterization of empty niosomes, produced by different methods and using different proportions of the formulation.

Three different methods have been explored for vesicle preparation (TLE-Paddle, TLE-Vortex and Reverse Phase method) and 6 proportions of the main excipients have been tested for niosomes formulation (Span®60, cholesterol and Solulan). Niosomes were characterized, in terms of particle size, homogeneity (polydispersity index PDI) and zeta potential.

The results obtained indicate that only the molar ratio composition had an impact on the characteristics of niosomas, since the properties of niosomes were not affected by the production methods. Among the tested niosome formulations, the 50:25:25 ratio of Span®60:Cholesterol:Solulan allowed to produce niosomes with the targeted characteristics, namely size (less than 200nm) and PDI (less than 0.2), followed by niosomes produced using Span®60:Colesterol:Solulan in the ratio 40:35:25.

In the future, the niosome surface will be functionalized with the mannose receptor and loaded with a selected antitumoral drug. The physicochemical properties of these vesicles will be characterized and their biological effect will be evaluated. Niosomes constitute promising targeted drug delivery systems. However, there are some limitations associated with their use, namely their translation for clinical use and the cost of production.

Keywords: Drug delivery, Niosomes, Methods of preparation, Niosomes' characterization

List of Acronyms

AFM	Atomic force microscopy
CHL	Cholesterol
CPP	Critical packing parameter
CQAs	Definition of the critical quality attributes
Cryo-SEM	Cryo-scanning electron microscopy
DCP	Dicetyl phosphate
DDS	Drug Delivery Systems
DLS	Dynamic light scattering
DPH	1,6-diphenyl-1,3,5-hexatrine
EDXD	Energy-dispersive X-ray diffraction
EE	Entrapment or encapsulation Eficiency
FAT	Freeze and thaw method
FF-TEM	Freeze fracture replication-electron microscopy
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
MLVs	Multilamelar Vesicles
NMR	Nuclear magnetic resonance spectroscopy
PBS	Phosphate buffered saline
PDI	Polydispersity index
RBBC	Rice bran bioactive compounds
Rpm	Rotations per minute
SAXS	Small-angle X-ray spectroscopy
SCFs	Supercritical fluid methods
SEM	Scanning electron microscopy
SOL	Solulan
SSL	Sodium stearoyl lactate
STM	Scanning tunneling microscopy
STR	Stearyl amine
TEM	Transmission Electron Microscopy
Tc	Gel liquid transition temperature
TFH	Thin Film hydration

TLH	Thin layer hydration
TLE	Thin Layer Evaporation
ULV	Unilamellar Vesicles

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1. Introduction

The development of different types of nanocarriers has been considerably revised by the scientific community, focusing mostly on polymeric nanoparticles, solid lipid nanoparticles, liposomes, micelles, dendrimers, carbon tubules, mesoporous silica and quantum dots. However, only a limited number of those carriers has reach the market and therefore is in clinical use (1–3).

The ultimate goals for designing an effective nano drug delivery system include the use of biocompatible and biodegradable materials; active targeted delivery of therapeutics to the pathological site without affecting the surrounding healthy tissue or organs; no premature or burst release; ability to load a significant amount of drug in order to achieve the desired therapeutic effect; controlled release of drug over an extended period to reduce dose frequency and improve patient compliance (1). Among the different drug delivery systems, niosomal carriers can fulfill most of the goals listed above.

Niosomes comprise the bi-layered structure of non-ionic surfactants agents. These thermodynamically stable bilayer structures are formed only when surfactants and cholesterol are mixed in a proper proportion, and the temperature is above the gel liquid transition temperature (4).

Niosomes are similar to liposomes in terms of their physical properties, structures and methods of preparation, being however developed using non-ionic surfactants instead of phospholipids, which are liposome main components (5,6). Niosomes constitute a promising delivery system able to overcome the problems associated with large-scale production of some classes of drug delivery systems, sterilization, insolubility, and rapid degradation of drugs (5,7,8)

Niosomes were first reported by researchers from L'Oréal (Clichy, France) for cosmetic applications in the 1970s and 1980s. Since then, niosomes have been extensively investigated for multiple applications in different fields, including pharmaceutical, cosmetic and food sciences (2,4). Within the pharmaceutical field, the most interesting studies are related to the capacity of niosomes to enhance drug delivery across the skin barrier (9). Therefore, niosomes are being explored for various types of topical applications, such as topical vaccine delivery and ocular delivery (1,10).

Drug targeting to specific sites of action provides several advantages over non-targeted drugs. These includes the prevention of adverse reactions of drugs on healthy tissues, enhancement of drug uptake by targeted cells and reduction of the dose required for the desired therapeutic effect (11). Most current anticancer agents do not greatly differentiate between cancer and normal cells. This leads to systemic toxicity and adverse effects, which greatly limit the maximum tolerated dose. Niosomes have been used for the targeted delivery of anti-cancer agents in order to decrease the overall toxicity by reducing adverse effects associated with the off-target effect of these drugs (3,12,13). To achieve an active targeting, niosomes have to be functionalized with a targeting moiety that will provide a preferential interaction with cancer cells. Our research project presented in the next chapter was specifically focused in the field of niosomes as antitumoral drugs delivery.

1.1. Niosome composition

In general, vesicles made of natural or synthetic phospholipids are called liposomes whereas those composed by nonionic surfactants (*e.g.* alkyl ethers and alkyl esters) and cholesterol constitute a nonionic surfactant vesicular system called niosomes (14–16). Niosomes or nonionic surfactant vesicles are now widely studied as an alternative tool to liposomes.

Because of their special geometry, niosomes can encapsulate hydrophilic and hydrophobic drugs in their structure. Entrapment of hydrophilic drugs in niosomes can occur in the central aqueous domain or it can be adsorbed onto bilayer surface, whereas the hydrophobic drugs enter the bilayer structure by partitioning into it. So, similarly to liposomes, niosomes are particularly useful vehicles for drug delivery of both hydrophobic and hydrophilic drugs (Fig.1) (4,14).

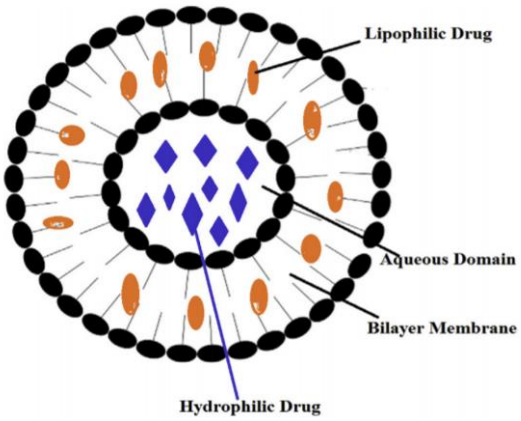

Figure 1.Bilayer structure of noisome. Reproduced from (4).

Niosomes mainly contain two types of components, a nonionic surfactant (main component) together with the additives and the hydration medium (1,2). The non-ionic surfactants form the vesicular layer, while the additives are added to the formulation in order to stabilize the niosomes (6). The most common additives found in niosomal systems are lipids, such as cholesterol (5,17).

1.1.1. Surfactants

Surfactants are amphiphilic molecules, which comprise a lipophilic tail and a hydrophilic head. They are classified according to the charges of their hydrophilic head groups, which are cationic, anionic, amphoteric and non-ionic (1,18) (Figure 2).

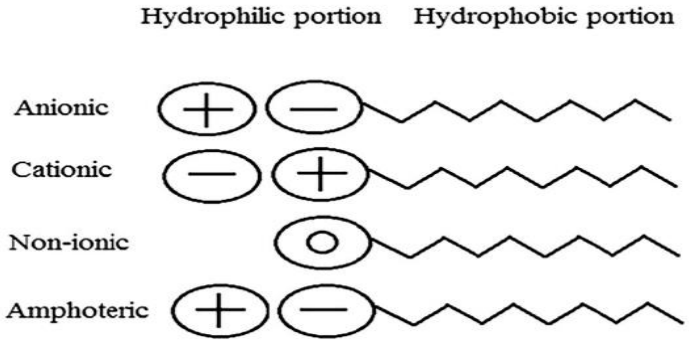

Figure 2. The different types of surfactants used in niosome formulation. Reproduced from (1).

Nonionic surfactants are the basic components of niosomes, which upon hydration form lamellar microscopic and nanoscopic vesicles. Nonionic surfactants are preferred as these compounds do not have a charged polar head, being thus less toxic, less irritant to cellular surface, less hemolytic and more biocompatible at physiological pH, compared with the other types of surfactants (1,2,4,14,18). An increase variety of non-ionic surfactants have been commonly used in the preparation of niosomes and are reviewed in the following sections (5,6).

1.1.1.1. Types of non-ionic Surfactants

Non-ionic surfactant vesicles can be prepared using different types of molecules, such as amino acids, fatty acids, amides, alkyl esters and alkyl ether surfactants. However, the last two are the most used surfactants and are summarized in Figure 3.

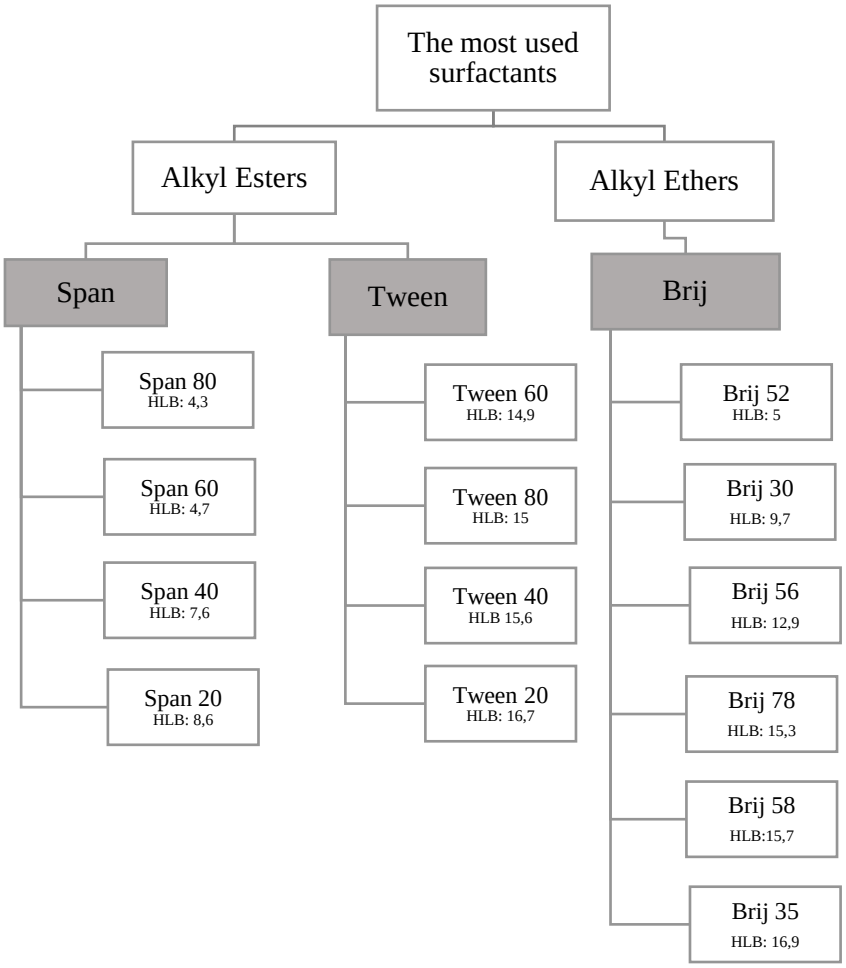

Figure 3.Surfactants mostly used in the development of niosomes (Adapted from (1)).

Alkyl Ethers

Alkyl ethers are good vesicle forming nonionic surfactants. Due to their high stability, alkyl ethers can be used to encapsulate proteins and peptides, although it has been shown that their encapsulation capacity is reduced when combined with cholesterol (14).

Brij surfactants are examples of alkyl ethers. The Brij 30 (Polyoxyethylene(4)lauryl ether) has a phase transition temperature less than 10°C and is able to form large unilamellar vesicles with high drug loading (1).

Alkyl Esters

Alkyl esters include sorbitan fatty acid esters (Span®) and polyoxyethylene sorbitan fatty acid esters (Tween®), which are non-toxic and non-irritating (5).

Tween® surfactants result from the reaction of polyoxyethylene with sorbitan fatty acid esters. They have higher hydrophilic-lipophilic balance (HLB) values compared with Span®

surfactants (2). Vesicles prepared by the polyoxyethylene sorbitan monolaurate (Tween® 20) are relatively more soluble than other surfactant vesicles (5).

Niosomes with Span® 40 have higher drug release rates than Span® 60 niosomes. This might be explained by the fact that niosomes demonstrate an alkyl chain length–dependent release and that longer chain lengths will lead to lower release rates (19). Span® 60 has a chain with 18 carbons, while Span® 40 has 16 carbons, and therefore Span® 40 will have a higher drug release.

Some recent studies report the synthesis and the characterization of new surfactants with peculiar physical–chemical properties, showing promising applications in the field of pharmaceutical colloid science. Bola and Gemini surfactants are the newer generations of surfactants that have been synthesized to produce niosomes with optimal properties (2).

Gemini surfactants have two hydrophobic chains and two hydrophilic head groups connected by spacers. This surfactant have a low critical micelle concentration, more stable, and are non-toxic, non-irritating and non-hemolytic (1,5,14,18).

Bola amphiphiles contains bipolar amphiphiles with two polar heads connected by one or two long hydrophobic spacers. Bola surfactants are of higher solubility, higher critical micelle concentration and lower aggregation number (1,18).

1.1.1.2. Properties of nonionic surfactant affecting niosome formulation

The selection of surfactants depends on their hydrophilic-lipophilic balance (HLB) value, critical packing parameter (CPP) and gel liquid transition temperature (Tc) (14).

Structure of surfactant (CPP)

The formation of bilayer vesicles and the geometry of the vesicles formed during the niosomal preparation also depends on the CPP. The geometry of the vesicles can be predicted by the CPP surfactant value. The CPP can be calculated using the equation displayed in Figure 4, considering the area of the polar head group, and the volume and length of the non-polar group.

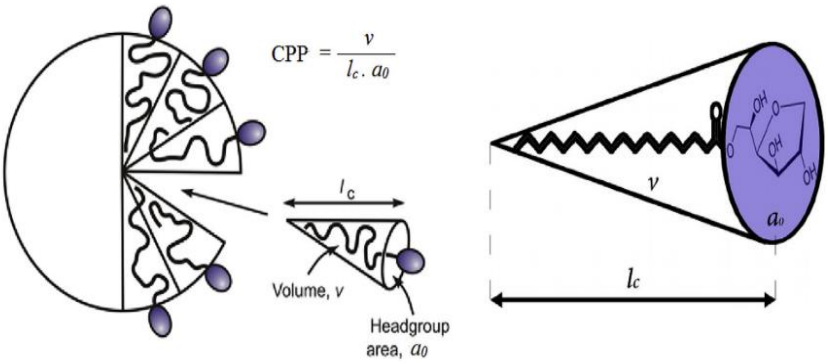

Figure 4. Critical packing parameter of an amphiphile. Reproduced from (4).

CPP is helpful in predicting the structure of the niosome, and the type of micellar structure formed can be ascertained according to criteria sumarized in Table 1.

Table 1. Critical packing parameter (CPP) predicting structure of niosome and type of micellar structure (1,4,5,17).

Geometry of the micelles	CPP value
Spherical	CPP<1/2
Bilayer	1/2<CPP<1
Inverted	CPP>1

A comparison between Tween® surfactants confirmed the influence of the surface area of the polar head. Tween® 21 and 20 have the same alkyl chain but different hydrophilic head groups, which influences the HLB value, the former being HLB 13.3 and the latter being HLB 16.7 (1,4,5,17).

Hydrophilic-Lipophilic Balance (HLB)

HLB is an empirical expression for the relationship of the hydrophilic and the hydrophobic groups of surfactants. Surfactants with a higher HLB value are more water-soluble than surfactants with a lower HLB (20–23). HLB value affects the size of the niosomes, as well as encapsulation efficiency (EE) of the active ingredient (24,25).

Surfactants with an HLB value of 6 or higher require the addition of cholesterol to form niosomes. Surfactants with an HLB outside of this range do not form niosomes (1,26).

The HBL also affects drug entrapment in niosomes, playing a key role in controlling drug entrapment within the niosome's vesicle. A surfactant with a HLB value in the range 14–17 is not suitable to produce niosomes, whereas one with a HLB value of 8.6 will lead to niosomes with the highest EE. Entrapment efficiency decreases as the HLB value decreases from 8.6 to 1.7 (14,27). The impact of HLB value on vesicle formation is summarized in Table 2.

Table 2. Impact of HBL value in niosomes' formation (Adapted from (23)).

HBL Value	Impact on formulation	References
14 – 17	Does not produce niosomes	(28)
8,6	Increase entrapment efficiency	(27)
1,7 – 8,6	Decreases entrapment efficiency	(14,28)
>6	Cholesterol is required for the formation of bilayer vesicle	(1,26)
Lower value	Cholesterol is required to increse stabilty	(26)

Gel liquid transition temperature (Tc)

Gel liquid transition temperature (Tc) of the surfactants is an essential factor that influences the formation of niosomes. It affects membrane fluidity, membrane permeability, stability and has a major role in EE (1).

Tc and the length of the alkyl chain of non-ionic surfactants are correlated with each other. Thus Span® 20 with a C9 chain is liquid at room temperature (27); Span® 40 with a C13 chain has a gel transition temperature of 46–47ºC; Span® 60 with a C15 chain has a gel transition temperature of 56–58ºC. Shorter alkyl chains have a lower Tc, which leads to the formation of "leaky" niosomes (12,28).

In addition, vesicles composed by higher molecular weight Spans are less leaky and more stable to osmotic gradients. Below this temperature, the permeability of the lipid bilayers is very low and so is the drug leakage. For example, Span® 60 has a high EE that may be related to high transition temperature (Tc of 50ºC) (4,14,17).

Tc is dependent on the degree of the unsaturated alkyl chain. Lack of saturation in the alkyl chain lowers Tc and increases chain fluidity and membrane permeability. Studies have shown that niosomes formed by surfactants with lower Tc are more flexible than those formed with a higher Tc (14,22,29,30).

The temperature of the hydration medium should be higher than the Tc of the surfactant, as this affects the formation of niosomes and could induce modifications in the bilayer (1,31).

1.1.2. Cholesterol

Steroids are important components of the cell membrane and their presence in membrane affect the bilayer fluidity and permeability. Cholesterol is a steroid derivative, frequently used for the formulation of niosomes, in a 1:1 molar ratio with the non-ionic surfactant (25). Cholesterol as such is not required for the formulation of niosome, but its use has a major impact on the physicochemical properties of these carriers, such as membrane permeability, rigidity, EE, ease of rehydration of freeze-dried niosomes, stability, storage time and their toxicity (4). The addition of cholesterol protects the drug molecules from the premature degradation and inactivation due to unwanted immunological and pharmacological effects. It was also found that the presence of cholesterol increases the niosome size significantly (1,5,18).

One of the most important factors affecting niosomes is the vesicle rigidity and fluidity. Cholesterol interacts with surfactant by forming hydrogen bonds between its hydroxyl groups and the alkyl chain of surfactant molecules, which enhances the stability of the bilayer. These interactions result in increased membrane cohesion and restriction of the movement of the bilayer acyl chains. It increases the transition temperature of vesicles and hence can improve their stability by altering the fluidity of chains in bilayers (1,19).

When present in sufficient concentration, cholesterol can eliminate the gel to liquid phase transition endotherm of surfactant bilayers, thus preventing the formation of aggregates by repulsive steric or electrostatic forces. As a result, the niosome becomes less leaky in nature (1,5).

The absence of gel to liquid phase has two main consequences:
i) delay on drug release, once the fluidity of the membrane is one of the critical factors;
ii) improve the encapsulation of hydrophilic drugs (21). However, for lipophilic drugs, higher concentrations of cholesterol in the formulation lead to lower EE, as cholesterol disrupts the regular linear structure of the niosomal membrane, which does not allow higher EE for lipophilic drugs (1,6).

The amount of cholesterol required in a formulation depends on the HLB value of the surfactants. The cholesterol increases the stability of the vesicles prepared with low HLB surfactants, while it helps the formation of bilayer vesicles when the surfactants present HLB

values higher than 6. In the case of surfactants with a HLB higher than 10, it will be required the use of higher amounts of cholesterol to prepare stable niosomes in order to overcome the effect of larger head groups. On the one hand, no cholesterol or small amounts of this excipient will be required for the preparation of niosomes using Span® 60 and 80, due to their low HLB values. On the other hand, Tween® 60 will demand for higher amounts of cholesterol to maintain membrane rigidity, as it present a higher HLB value (4,14,20).

1.1.3. Charged molecules

Charge inducing agents also play an essential role in stabilizing bilayer membranes by imparting either negative or positive charges to the surface of the niosomes, thus preventing aggregation by electrostatic repulsion (1). Niosomes with a zeta potential higher (i.e. surface charge) than +30mV or lower than −30 mV have been widely reported as having an acceptable stability; zeta potentials between |5| and |15| mV represent the region of limited flocculation, while values between |5| and |3| mV correspond to the maximum flocculation. Thus, particle aggregation is less likely to occur for charged particles (high zeta-potential) due to their electric repulsion (1,2,32).

Negatively-charged ionic compounds, such as dicetyl phosphate (DCP) or phosphotidic acid, and positively-charged compounds, like stearyl amine (STR), sodium stearoyl lactate (SSL) or stearyl pyridinium chloride, are charge inducing agents commonly used in the preparation of niosomes. Generally, these charged molecules are added to the formulation in an amount of 2.5–5 mol% (25,33). However, amounts of charge inducing agents beyond the limit will prevent the formation of niosomes (1,5,21).

As these charged inducing molecules improve the stability of these vesicles, their use will also lead to higher drug EE. For example, the addition of sodium stearoyl lactate (SSL) as charge inducing molecule increased niosome's EE due to the electrostatic interaction obtained between the SSL negatively charged group and the drug positively charged residue (4,34).

Manosroi et al. (35) reported that the interaction between charges can influence niosome size. Positively charged drugs neutralize the charge of anionic molecules and therefore niosomes will have smaller sizes than cationic vesicles due to the repulsion obtained between the cationic charges of the drug and the added molecules.

Charged molecules can also be added to prepare charged niosomes towards skin permeation enhancement (33), as well as looking at hybrid niosomal complex formation (2).

1.1.4. Hydration medium

An additional important component required for niosome preparation is the hydration medium. Usually, phosphate buffer is used as hydration medium. It should be noted however that the pH of the buffer depends on the encapsulated drug solubility (8,14). For example, pH 5.5 phosphate buffer was used in the preparation of ketoconazole niosomes whereas pH 7.4 phosphate buffer was used in the preparation of meloxicam niosomes (36).

Understanding the physicochemical properties of the formulation components as well as their effects on niosomes, are essential steps in the preparation of a customized niosome, with all the specific desired properties. The major factors affecting niosomes' properties will be discussed in the following Section.

1.2. Factors affecting the physicochemical properties of niosomes

1.2.1. Surfactant structure

As already discussed in the section above, HLB, gel transition temperature and CPP of a surfactant are important factors influencing physicochemical properties of vesicles throughout the process of nisome' development.

The geometry of the vesicles formed during the niosome's preparation also depends on the CPP, as explained in Table 1 (5,19).

Phase transition temperature provides the highest entrapment rate, while higher monomer surfactant hydrophobicity leads to smaller vesicles, as shown in Figure 5. This result might be anticipated since the surface free energy decreases with increasing hydrophobicity (27). Also related to changes in niosomes size, published literature refers that the stearyl chain (C18) surfactants (span® 60 and brij 72) leads to vesicles significantly larger than those developed using the palmityl chain (C16) surfactant (span® 40). This outcome can be explained by the alkyl chain length, because a longer chain will lead to a wider bilayer (4,37).

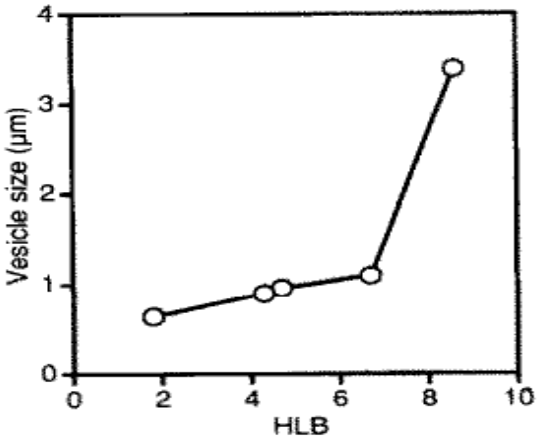

Figure 5. Effect of HLB on vesicle size (27)

1.2.2. Effect of cholesterol content

Cholesterol influences the physical properties and the structure of niosomes at the same level as it affects biological membrane physical properties. The effect of cholesterol in lipid bilayers is mostly related to membrane cohesion and mechanical strength, as well as permeability to water. Cholesterol can influence the properties of niosomes in the following aspects:

i) Reduce the flexibility of the membrane, which is very important under severe stress conditions (14);

ii) Reduce the permeation of drugs across membranes (1,19);

iii) Decrease the drug release rate (5);

iv) Improve the encapsulation of hydrophilic drugs, as shown in Figure 6. In fact, it has been shown that niosomes containing 60% of cholesterol present has higher EE. However, higher lipid content (80 mg/ml) may result in a saturable environment where the successful formation of the vesicles may be affected (21,38,39);

v) Improve the stability of niosomes by increasing the transition temperature (1).

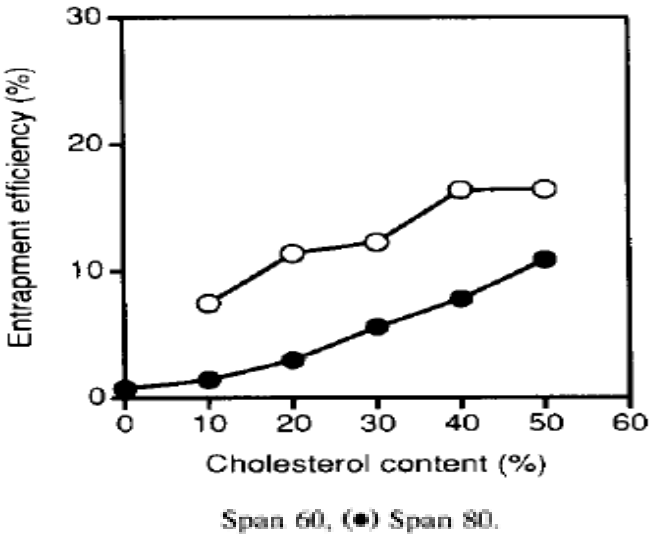

Figure 6. Effect of the cholesterol content on the entrapment

1.2.3. Surfactant and lipid amount

Generally, the maximum amount of surfactant and lipid used for preparing niosome is 10–30 mmol/L (1–2.5% w/w), respectively. Alterations in the surfactant:water ratio during the hydration step may affect the structure and properties of the niosomes. As the surfactant/lipid level increases, the amount of drug to be encapsulated also increases leading to higher viscosity (4, 12, 16).

The effect of the total lipid concentration on the amount of drug entrapped has been addressed as well. Mokhtar *et al.* (37) reported that the EE% of flurbiprofen and total lipid concentration were directly correlated. Similar results were obtained by *Yoshioka et al.* (27) who reported a linear increase in the EE of 5(6)- carboxyfluorescein, while increasing the total lipid concentration.

1.2.4. Properties of drugs

The drug entrapment in niosomes is affected by molecular weight, chemical structure, hydrophilicity, and lipophilicity. The size of the vesicles may increase, due to drug interaction with the surfactant head groups, which may increase the polymer charge and thus cause repulsion of the surfactant bilayer, thus increasing the vesicle size (5,12,35).

Regarding the influence of the drug concentration on the EE, several studies have shown that higher drug concentrations lead to higher EE (14,41). More recently, it was shown that

high drug loadings decreases the EE as it interferes with vesicle formation (4). The effect of encapsulated drug on niosome properties is also shown in Figure 7 (18).

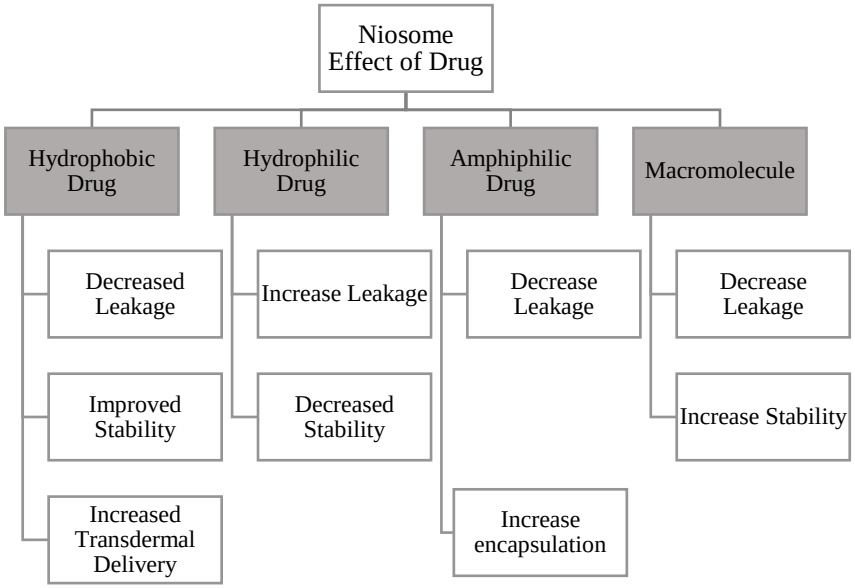

Figure 7. The effect of the nature of the encapsulated drug on the properties of the niosome dispersion (18,45)

1.2.5. Hydration temperature

Hydration temperature affects the structural properties of niosomes, their shape and size. Changes in the temperature can also affect the formation of vesicles. Ideally, the hydration temperature for niosome formation should be above the gel to liquid phase transition temperature, once temperature affects the assembly of surfactants into vesicles (4,5,12,37).

Polyhydral vesicles of C16:solulan C24 (91:9) were formed at 25°C, being however converted into spherical vesicles around 45-48°C. While cooling from 55 to 49°C, the vesicles produced a cluster of smaller spherical niosomes, and changed to a polyhedral structure at 35ºC. In contrast, vesicles formed by C16:cholesterol:solulan C24 (49:49:2) showed no shape transformations on heating or cooling conditions (5,31,43,44).

Maryam (45) reported that increasing the temperature and time (from 10 to 25 min) the number of giant oligolamellar, multishell and multilamellar niosomes increases. The optimal time and temperature were found to be 25 min and 70–75°C.

1.2.6. Hydration Time

Yeo et al. (38) studied the effect of hydration time and hydration volume on EE and vesicle size. These authors reported that short hydration time produces larger vesicles with low EE. These studies suggest 60 min as an optimal hydration time and 5 ml hydration volume for full hydration and formation. Longer hydration time produces small-sized vesicles and increases EE (46).

1.2.7. Effect of volume and pH of the hydration medium

The pH of the hydration medium also affects the EE of the drug (4).

For example, flurbiprofen shows higher entrapment at acidic pH with a maximum EE of 94.6% at pH 5.5. The EE of flurbiprofen increases as the pH decreases from 8 to 5.5, and the EE decreases significantly at pH 6.8. Higher EE of flurbiprofen at lower pH is possibly due to its ionizable carboxylic acid group (4,14,41).

Rukmani and Sankar (46) prepared niosomes in phosphate buffer saline at pH 7.4, and observed higher drug leakage for higher hydration medium volume.

Improper selection of all these factors related with the hydration medium may result in the formation of fragile niosomes, with low drug loadings.

1.2.8. Resistance to osmotic stress

When a hypertonic salt solution (e.g. KCl or glycerol) is added to a niosomal suspension, the size of niosome decreases (5,47–49).

Studies have been made to understand the influence of osmotic upshift on niosome development.
When the membrane was relatively permeable to the osmolyte (for example, glycerol), the vesicle returns to its normal conditions within seconds or minutes. However, when the osmolyte is relatively impermeable (*e.g.* KCl), the vesicle remains in the shrinking stage for hours (4,50).

1.2.9. Method of preparation

Niosome preparation method may affect its size and EE (4,5,39,56).

Ethanol injection method produces niosomes smaller than those prepared using the thin-film hydration method. Small size niosomes can also be produced from reverse-phase evaporation and microfluidization (4,51).

The average size of niosomes prepared by the hand-shaking process leads to niosomes with diameters in average higher (2.7 nm) than those developed by the ether injection method (1.5nm). Niosomes produced by the ethanol ejection method have a small diameter, probably due to the fact that the production technique requires the passage of cholesterol and Span through the needle orifice into the drug solution (4,5,48,52,53).

Overall, the published literature reported that the EE of niosomes formed using reverse-phase evaporation technique has shown the best EE in comparison to the niosomes formed using thin-film hydration technique, freeze and thaw method and de-hydration and rehydration method (34).

The following section described these different methods explored for producing niosomes in more detail.

1.3. Preparation methods

The general methodology employed for the preparation of niosomes involves five basic stages:

1. Evaporation to produce a lipid film;
2. Lipid dispersion in the hydration medium;
3. Niosomal suspension purification;
4. Niosome size reduction (optional) (14,18,54).

However, there are variants of this general method, which are described in Table 3.

A large number of methods use organic solvents (*e.g.* chloroform, methanol, diethyl ether and acetone), and residues of these toxic solvents may remain in the final niosomes preparation and contribute to their potential toxicity. Therefore, it would be much preferable to avoid the use of these solvents (15,55,56). The most recently selected methods consider this important point and have the advantage of not using organic solvents.

Table 3. Niosome preparation techniques.

Preparation method	**Method description**	**Advantages**	Disadvantages	References
Thin Film hydration (TFH) Hand shaking method | Thin Layer Evaporation (TLE)	Surfactants and cholesterol are dissolved in a round-bottomed flask followed by evaporation of the organic solvent to form a thin film on the bottom of the flask. This film is then hydrated with aqueous medium with gentle mechanical shaking, at a temperature above the transition temperature of the surfactant, to obtain multilamellar vesicles which are further treated for size/lamellae reduction.	Easy technique for laboratory scale production.	Use of organic solvents.	(2,4,17)
Ether injection method	Surfactans and drug are dissolved in diethyl ether and injected slowly into an aqueous phase, which is heated above the boiling point of the organic solvent. This produces large unilamellar vesicles (LUVs) and can be further treated for size/lamellae reduction. The use of other alcohols has also been reported. The selection is based on their toxicity, water-solubility, viscosity and dissolving power towards lipids and drugs.	Easy technique for laboratory scale production.	No applicability for thermosensitive drugs.	(2,17,57–61)
Reverse phase evaporation method	The surfactants are dissolved in an organic solvent and an aqueous phase containing the drug is further added. The resulting two-phase system is then homogenized, and the organic phase evaporated under reduced pressure to form niosomes dispersed in the aqueous phase.	High drug entrapment efficiency (EE).	Use of organic solvents.	(5,14,60,62)
Trans-membrane pH gradient drug uptake process	After film formation (TLE), using the same method as in Thin Film hydration, the hydration of this film is carried out with a citric acid solution at pH 4 by vortex mixing. The resultant vesicles then undergo a freeze and thaw process for three cycles and sonication. An aqueous drug solution is then added and vortexed. The pH of this solution is adjusted to 7-7.2 by adding a buffer solution, and heated at 60°C.	High EE.	Use of organic solvents.	(2,14,48,57,63)

Microfluidization method	Drug and surfactant are dissolved and pumped under pressure at a rate of 100 ml/min from a reservoir to an interaction chamber packed with ice. The solution is then passed through a cooling loop to remove the heat produced during microfluidization to form niosomes. This method produces niosomes of smaller size with great uniformity.	No use of organic solvents, which are expensive, difficult to remove and unsafe.	No applicability for thermosensitive drugs.	(2,4,14,59,64)
Bubble method	Surfactants and cholesterol are dispersed at 70°C and homogenized for 15 seconds and immediately after, nitrogen gas is supplied to this mixture, producing LUVs. Small unilamellar vesicles will be further obtained once LUVs are subjected to size reduction.	No organic solvent.	No applicability for thermosensitive drugs.	(2,5,17,18,48)
Niosome prepared using micelle solution and enzymes or Enzymatic method	Niosomes can be prepared with a mixed micellar solution using enzymes. In this method, esterases break the ester links of polyethylene stearyl derivatives leading to the formation of breakdown products, such as cholesterol and polyoxyethylene, which can form multilamellar niosomes with the addition of dicetyl phosphate and other lipids.	No organic solvents.	The active ingredient may be degraded by enzymatic degradation.	(1,2,14,18)
Lipid injection method	Surfactant and cholesterol are melted and then injected into a highly agitated heated aqueous phase containing dissolved drug molecules to form a niosomal suspension.	No organic solvents.	No applicability for thermosensitive drugs.	(1,2)
Formation of niosomes from proniosomes or Proniosomal method	Another method of producing niosomes involve coating a water-soluble carrier (*e.g.* sucrose stearate, maltodextrin or mannitol) with nonionic surfactants. The result of the coating process is a dry formulation. The water-soluble carriers are covered by a thin layer of surfactant, known as “proniosomes”. This formulation is rehydrated under agitation in hot aqueous media to form a niosomal suspension.	No organic solvents. Better physical stability. Increase EE.	Complex process. Complete drug entrapment may not be possible during hydration.	(2,65–67)
Supercritical fluid methods (SCFs)	Supercritical fluids (SCFs) methods have emerged as a green and novel technique. The Most commonly used SCF is the carbon dioxide. The preparation of niosomes using supercritical fluids has been described by Manosroi (68).	No organic solvent. Easy scaled up. Uniform size. High Stability.	Special equipment required.	(2,57,68,69)

The single pass technique	This is a patented technique involving a continuous process that comprises the extrusion of a solution of a lipid-containing drug through a porous device and subsequently through a nozzle. It combines homogenization and high pressure extrusion to produce niosomes with a narrow size distribution in the range 50–500 nm.			(4,14)
The Handjani: Vila method	This method involves the mixing of cholesterol and surfactant with the aqueous solution containing the active substance. The resultant mixture is homogenized using ultracentrifugation or agitation, while the temperature of the process is kept controlled.	No organic solvent.		(14,18,70)
Heating method	Surfactants and some additives, such as cholesterol are separately hydrated in PBS (pH = 7.4) under nitrogen atmosphere. After hydration, the cholesterol solution is heated for 1 h at around 120°C. The temperature is lowered to 60 °C and the other components, surfactants, and other additives, are added with continuous stirring. Niosomes obtained at this stage are left at room temperature for 30 min and then kept at 4–5°C under nitrogen atmosphere until use.	Flexibility for the entrapment of various drugs and, respecting temperature sensitivities.		(4,17,55)
Dehydration-rehydration technique	These vesicles are first prepared by a thin-film hydration technique, and then frozen in liquid nitrogen and freeze-dried overnight. Powder niosomes are hydrated with phosphate buffer saline (pH – 7.4) at 60°C.	High EE.	Heterogeneous vesicles formation with low reproducibility.	(17,50,57)
Freeze and thaw method (FAT)	Niosomes are prepared by TFH method, being further frozen in liquid nitrogen for 1 min and thawed in a water bath at 60°C for another 1 min.	Freeze and thaw cycle shrinks the niosomes, when prepared by unsaturated surfactants.	Use of organic solvents. Freeze and thaw cycle reduce EE.	(4,17,50)

1.4. Niosome purification

Purification of niosomes is an essential step, as complete encapsulation of drug molecules in niosomes is seldom possible regardless of optimization of the drug loading processes (2). Usually, a large amount of drug is left unentrapped during the hydration phase. Also, both methods are used to decrease the concentration of the residual solvents. Purification methods include dialysis, gel filtration, and centrifugation (1,4,55).

1.4.1. Dialysis

The dialysis method is a process based on diffusion and osmosis that depends on the movement of both solute and solvent across a semi-permeable membrane. The niosomes are filled in a dialysis bag and the free drug is dialyzed using a saline solution (phosphate buffer). The concentration of the drug in the saline solution is determined by spectrophotometry (2,15,16,71–73).

1.4.2. Gel filtration

Purification of niosomes from the unencapsulated drug can be carried out by gel filtration chromatography on Sephadex G75, G50 or G25 (15,74–77). Gel-filtration chromatography is a popular and versatile technique that allows the effective separation of free molecules in high yield (2).

1.4.3. Centrifugation/ultracentrifugation

It is the most preferred method for the purification of niosomes (4,78–81). This method is used to separate the unentrapped genetic material from niosomes by gradient density centrifugation. These methods can be applied to a variety of solutes and more than 90% recovery can be achieved without dilution of niosome preparation (1,82). Some authors combined this approach with the gel filtration chromatography using Sephadex (83,84).

1.5. Reduction of size of niosomes

To ensure the desired size, lamellarity, and homogeneity properties of niosomes, post formation processing is required. The most common methods for post formation processing are sonication and extrusion (15,85).

1.5.1. Sonication

Sonication is used to reduce the size of the vesicles. This niosomal suspension, which is generally composed for Multilamellar Vesicles (MLVs), is sonicated either by probe sonicator or bath sonicator, which in turn leads to the formation of unilameller vesicles. The sonication time and cycles can be adjusted taking into account the vesicle size wanted (17,48,51,55).

1.5.2. Extrusion

The membrane-extrusion method reduces the size of the liposomes (large Unilamellar vesicles (ULVs) or MLVs) by passing them through a membrane filter with a defined pore size (mean pore size 0.1mm) (5,51,55).

1.6. Characterization studies

The characterization studies include parameters such as size, distribution, zeta potential, morphology, EE, and i*n vitro* release behavior. These studies determine the quality of the niosomes in formulation development and their application in future clinical studies (17). All these characteristics are summarized in Table 4 and discussed in more detail below.

1.6.1. Particle size and size distribution

Particle size is a fundamental parameter in the characterization of niosomes as it provides information on physical properties and formulation stability (17). Niosome size can range from around 20 nm to 50 μm (86). Niosomes can be characterized using several techniques summarized in Table 4. However, the main techniques used to measure niosomes size are dynamic light scattering (DLS) and microscopy.

DLS is also known as photon correlation spectroscopy (2). This method is rapid and non-destructive, and only a small amount of sample is required. It can be used to measure particles in the size range of 3–3000 nm. The limitation of DLS is that it does not provide any information on the shape of the niosomes (2,14,18).

Particle size measured by the transmission electron microscope (TEM) is smaller than the DLS method because of the different measurement principles used by these two methods. In DLS technique the niosomes are hydrated, in a suspension, and in TEM technique the niosomes are dry (51). Electronic microscopic techniques are also used to determine the size

of niosomes, DLS and microscopic techniques are sometimes used in combination to produce more reliable results (4,17).

The poly-dispersity index (PDI) is an indication of the distribution of niosome size, with PDI value of less than 0.2 indicating an homogeneous sample (87). Niosomes' size distribution and PDI can be measured by using DLS particle size analyzer, such as Mastersizer and Zeta Sizer (5).

1.6.2. Morphology

Microscopic techniques are used to study the morphology of niosomes and are summarized in Table 4. Most of these methods allow to study the size and shape of the niosomes, and in particular Transmission Electron Microscopy (TEM) allows to determine the lamellarity (1,2,15,50,51).

1.6.3. Zeta potential

The zeta potential, which is also known as surface charge, provides essential information in determining the physical stability of niosomes. Generally, charged niosomes are more stable against aggregation and fusion than uncharged vesicles, and that is why niosomes are prepared by the inclusion of the charged molecules. Niosomes repel each other as the charge keeps them stable by preventing aggregation and fusion (5,17,86,89,90).

The methods used to measure the zeta potential (Table 4), and the magnitude of zeta potential provides an indication of the degree of electrostatic repulsion between two adjacent particles (1,4,5,15). Systems with the zeta potential value of ±30 mV or higher are considered to be stable (1,2,37).

1.6.4. Number of lamellae

Niosomes are either present in a single layer (unilamellar) or multiple layers (multilamellar) (1,4). Different techniques can be used to determine number lamellae (Table 4) (2,5).

The Small-angle X-ray spectroscopy (SAXS) can be coupled with energy-dispersive X-ray diffraction (EDXD) to characterize the thickness of niosomal bilayer (1,2,4,91–93).

1.6.5. Membrane Rigidity

The fluidity of the niosomal membrane allows membrane deformation without disrupting bilayer integrity (1,2). Membrane rigidity can be measured by means of the mobility of fluorescence probes that can be used as a function of temperature and/or time. In order to get

an understanding of the packing structure of the niosomal membrane, fluorescence polarization can be utilized to characterize the microviscosity of the membrane (1,2,4,5,94).

Examples of these fluorescence probes include 1,6-diphenyl-1,3,5-hexatrine (DPH) and pyrene. Both fluorescence probes can provide a wider picture of the bilayer characteristics, as the DPH gives an indication of lipid order, while pyrene shows lateral diffusion (95,96).

The fluidity of surfactant vesicular membrane is determined by fluorescence anisotropy, which correlates to microviscosity near the probe. In fact, the fluorescence anisotropy values are inversely proportional to membrane fluidity. A high degree of fluorescence anisotropy represents a high structural order and/or low membrane fluidity (4,97,98).

1.6.6. Vesicle Stability

The main problems associated with storage of vesicles are aggregation, fusion and leakage of drug (14). Stability studies are performed to evaluate storage feasibility, by exposing niosomes to different conditions of temperature (4ºC, room temperature and 40-45ºC) for several months (15,26,74,99). Niosomes are also exposed to various humidity and light (UV) conditions, which can promote photodegradation. Stability is determined by the periodic monitorization of parameters like size, shape, and EE. Variations in more than two parameters can indicate instability (1,4).

Stability of the vesicular system constitutes an issue and concerns physical, chemical and also biological properties of carriers. Therefore, the stability of niosomes in the presence of gastrointestinal enzymes can also be characterized, by exposing the drug and drug-loaded niosomes to different gastrointestinal enzymes (*e.g.* pepsin, trypsin, and chymotrypsin) (4). It has been shown that niosomes protect the drug from degradation by gastrointestinal enzymes (34).

Stability of niosome is influenced by the entrapped drug, its concentration, type of surfactant and cholesterol content. Therefore, these factors are adjusted in order to optimize stability (5,17).

1.6.7. Entrapment efficiency (EE)

EE is described as the number of drug molecules that have been successfully entrapped within the vesicles. It is defined as the percentage of the added drug that remains entrapped within the niosome, and can be expressed by the following equation (1,5):

$$E = (\text{Amount of drug entrapped} \div \text{Total amount of drug added}) \times 100\%$$

The amount of drug entrapped refers to the actual amount of drug molecules that has been successfully enclosed, which may be calculated by subtracting the amount of unloaded drug from the total amount of drug added (4,14).

For the determination of EE, the un-entrapped drug or free drug molecules, need to be first separated using suitable methods, such as dialysis, filtration or centrifugation (15,71). Different methods can then be used to determine the concentration of the loaded drug (Table 3).

1.6.8. *In vitro* drug release

The *in vitro* release behavior of the niosomes is a fundamental parameter that can be affected by many factors, such as drug concentration, hydration volume, and nature of the membrane (1).

The *in vitro* release studies are carried out by dialysis through a semipermeable membrane. In this dialysis membrane method, the niosomes are added into this dialysis bag, which is then placed in a vessel containing the dissolution media, which is usually PBS. This whole assembly is kept on a magnetic stirrer at a controlled temperature of 37°C. A sample solution is taken from the beaker at specified time intervals and replaced with fresh dissolution media. The samples were analyzed for the concentration of drug at its wave length (37,40,80,100–104).

Franz diffusion cells have also been used to study the release behavior of niosomes. This is where the dialysis membrane is placed between the donor- where are the niosomal suspension- and the receptor compartment of the apparatus. The receptor compartment contains PBS at pH 7.4 and the whole system is maintained at 37°C. Samples are collected from the receptor compartment at defined time intervals and replaced with the same amount of release medium (1,40,65,105,106).

1.6.9. Tissue Distribution/*In Vivo* Study

Tissue distribution profile has been studied using suitable animal models. To study the distribution pattern, drug-loaded niosomes are administered to animals, namely through the oral route. Then, animals are sacrificed and various tissues, like liver, kidney, heart, lungs and spleen are removed, washed with buffer, homogenized and centrifuged. The supernatant is after analyzed for the drug content (5,14).

Blood samples are collected at predetermined intervals and the supernatant is used to determine serum drug concentration by an adequate assay (4,107).

Table 4. Characterization techniques for niosomes.

Parameters	Methods	References
Particle size and size distribution	-Dynamic light scattering (DLS) -Transmission electronic microscopy (TEM) -Scanning electron microscopy (SEM) -Freeze fracture replication-electron microscopy (FF-TEM) -Optical microscopy techniques -Master Sizer -Zeta Sizer	(17,102,108)
Morphology	Preferentially for liquid samples -TEM -Negative-stanning transmission electronic microscopy (NS-TEM) -FF-TEM Preferentially for liquid state samples -SEM -Cryo-scanning electron microscopy (Cryo-SEM) -Atomic force microscopy (AFM) -Scanning tunneling microscopy (STM)	(1,2,16,17,86,88,91,109)
Zeta potential	-Laser doppler anemometry -Mastersizer -Zeta Sizer -Microelectrophoresis -pH-sensitive fluorophores -High-performance capillary electrophoresis -DLS	(1,4,5,15,50,55,60)
Membrane Rigidity	-Fluoresce polarization	(1,2,4,94,97,98)

Number of lamellae	-AFM -Nuclear magnetic resonance spectroscopy (NMR) -Small-angle X-ray spectroscopy (SAXS) -Electron microscopy	(1,2,5,91–93)
Vesicle stability	-DLS -Microscopic techniques -Turbiscan	(15,26,74,99, 111)
EE	-UV-spectrometer -High performance liquid chromatography (HPLC) -Fluorescence	(1,15,71,112,113)
In vitro release	-Dialysis -Franz diffusion cells	(37,40,65,80, 101–106)

1.7. Niosome Applications

Niosome development frequently aims to overcome drug formulation limitations in order to improve active pharmaceutical ingredient stability and loading, as well as prolong its release and therefore modulate its bioavailability (5,57). Major therapeutic applications of niosomes include anti-tumor drug delivery, diabetic therapy (114,115), diagnostic imaging (5), anti-inflammatory effect, localized psoriasis (116), alopecia (117), topical fungal infection (118), auto-imune disorders (2), leishmaniasis (119), vaccine immunizations (120), as well as lung, liver and brain targeting (5,60,73). Different delivery routes can be used for niosomes administration, including oral, topic, intravenous, pulmonary, transmucosal and vaginal routes of admnistration (4,15,121). However, niosomes are most frequently used as transdermal and ocular drug delivery systems.

Despite being extensively investigated, only a limited number of niosomal formulations have been able to reach the market. These include niosomes loaded with a specific medicine (dorzolamide) and anti-aging product by Lancome or L'Oreal (4,122,123). Several niosomal formulations (Table 5), are in clinical development.

Table 5. Niosomal formulations in clinical trials (57).

Vesicular System	Drug	Application
Niosomal gel	Gallic acid	Skin aging
	Griseofulvin	Tinea circinata
	Hydroxychloroquine	Oral lichen planus
	Methotrexate	Psoriasis
	Urea	Psoriasis
Proniosomal gel	Vinpocetine	Cerebrovascular disorder
	Finasteride	Androgenetic alopecia
Niosomal gel and cream	Rice bran bioactive compounds (RBBC)	Skin aging

2. Major goals of this project

Cancer cells frequently display glycans at different levels of expression or with structures fundamentally different from those observed on normal cells. In particular, high-mannose type oligosaccharides are expressed in the surface of various cancer cells, contrary to normal cells where these oligosaccharides are not commonly found (124,125). Because mannose is prevalent as the terminal non-reductive sugar of glycans in cancer cells, terminal oligomannosides are attractive therapeutic targets. Recently, it was found that mannan-binding lectins presented anticancer activity in animal model of human cancers (126), such as colon and breast cancer (127), by targeting the mannose residues on cancer cell surface and inducing apoptosis.

Therefore, biomimetic synthetic receptors for mannosides represent an attractive tool for the development of DDS. Moreover, DDS based on functionalized nanovesicles might promote the recognition of high-mannose glycans by multivalent presentation of the synthetic receptors (128).

The main objective of this project concerns the development of a novel actively targeted DDS for cancer therapy, obtained by functionalizing non-selective anticancer drug (*e.g.* doxorubicin) - loaded nanovesicles with biomimetic synthetic receptors that recognize mannosides (Figure 8).

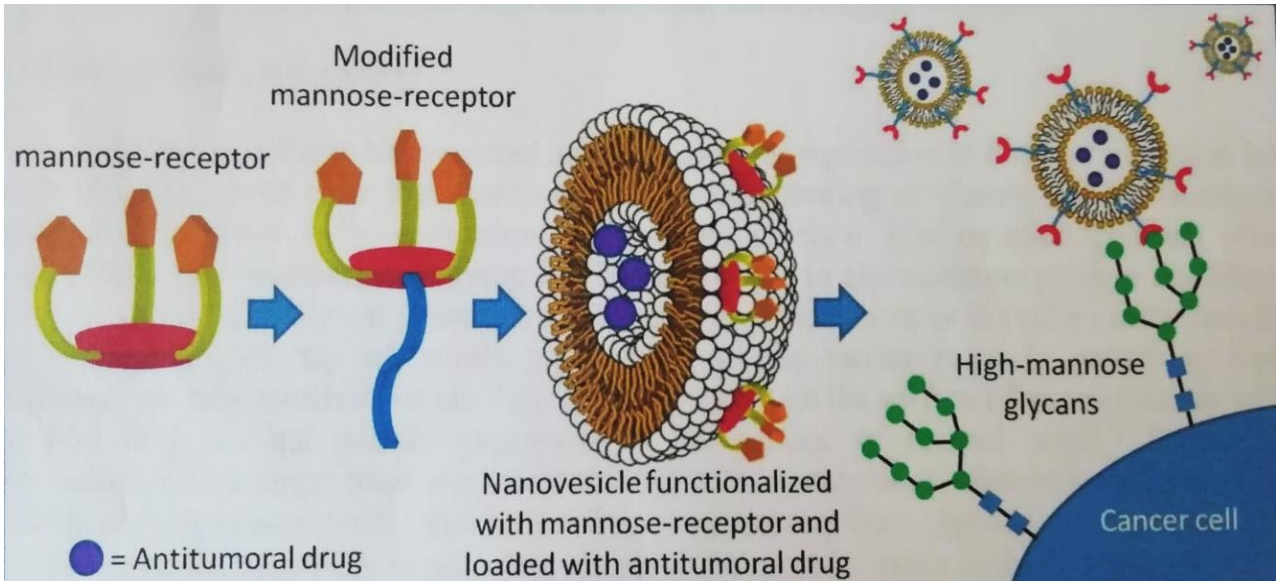

Figure 8. Graphical representation of the main topic of the project

In this context, the project is structured into two interconnected lines of research:

i) Rational design, synthesize and characterize modified biomimetic synthetic receptors for mannosides suitable for the functionalization of the nanovesicles;

ii) Design and characterize suitable nanovesicle formulations bearing the artificial receptors exposed on the surface;

In this project, we will focus on the second line of research that was organized into the following phases:

a) selection of the most suitable class of nanovesicles and the nature of their composition, starting from conventional liposomes;
b) design of the most suitable method to develop the selected nanovesicles;
c) preparation and characterization of functionalized empty nanovesicles by different methods and molar composition ratios;
d) selection of the most suitable method and ratios to produce functionalized niosomes for mannose-target antitumoral drugs delivery.
e) selection of the antitumoral drug to be loaded, chosen among wide-spectra chemotherapies;
f) preparation and characterization of functionalized nanovesicles loaded with the anticancer drug in terms of both morphological and technological properties.

Due to the setbacks imposed by the Covid-19 outbreak, it was not possible to finish the laboratory work previously planned, and we only achieved some results related to phase c). Consequently, this monograph will focus on the laboratory research work that was possible to perform, regarding the preliminary results obtained on the preparation and characterization of empty nanovesicles that will serve as an illustration of the proposed research project.

3. Materials and methods

3.1. Materials

Solulan C24 (Poly-24-oxyethylene cholesteryl ether, SOL) was kindly donated by Lubrizol (Cleveland, Ohio, USA). Cholesterol (CHL), sorbitan monopalmitate (Span® 40, HLB 9.8), sorbitan stearate (Span® 60, HLB 4.7), sorbitol was provided by Sigma-Aldrich (Milan, Italy). Phosphate buffer solution pH 7.4, 0.1 M (PBS) was prepared according to Eur. Pharm. 6th Ed.. Distilled water was used throughout the study. All other reagents and solvents were from Sigma Aldrich (Milan, Italy).

3.2. Preparation of Niosomes

Niosomal suspensions were prepared by four different methods, using Span® 60 as the main surfactant. Cholesterol (CHL) and Solulan (SOL) were also included in the niosomal formulations, at different molar ratios.

Table 6. Molar compositon ratios (Mol%) used to produce niosomes.

Formulation	Mol%
Span60:CHL:Solulan	50:40:10
	40:50:10
	35:40:25
	50:25:25
	25:50:25
	40:35:25

3.2.1. Thin layer evaporation (TLE)-paddle stirring method

Different amounts of Span® 60, CHL and Solulan were dissolved in 10 mL of chloroform in a round-bottom flask at different molar ratios (Table 6). After mixing, the solvent was evaporated under reduced pressure and constant rotation (150 rpm), for 30 minutes, to form a thin lipid film. Then, the layer was hydrated with 20 mL of PBS under stirring by a paddle at 2000 rpm for 30 minutes, while heating in a water bath at 65ºC.

The temperature of 65°C was selected as it is above the phase transition temperature of Span® 60, which is the component of the lipid mixture with the highest transition temperature (50°C).

3.2.2. Thin layer evaporation (TLE)-vortex method

Different amounts of Span60, CHL and Solulan were dissolved in 10 mL of chloroform in a round-bottom flask to obtain the different molar ratios. After mixing, the solvent was evaporated under reduced pressure and constant rotation (150 rpm), for 30 minutes, to form a thin lipid film.

After the hydration of the thin layer with 20 mL of PBS, 4 cycles of heating (3 minutes at 65ºC) and vortex mixing (3 minutes) were performed.

3.2.3. Reverse Phase method

Different amounts of Span60, CHL and Solulan were dissolved in 10 mL of chloroform in a round-bottom flask, to achieve the anticipated different molar ratios. After mixing, 20 mL of PBS were added. This dispersion was sonicated for 3 hours (Eurosonic 44 ultrasonic bath, Anwendungstechnik, Offenbach, Germany) to form a Water/Oil emulsion. Chloroform was then removed by rotary evaporation for 30 minutes, at 65ºC and 150 rpm.

After preparation, despite the different techniques, all the niosomal suspensions were subjected to ultracentrifugation for 15 minutes at 4000rpm, and treatment for size reduction, as described in the following section.

3.3. Size reduction of niosomes

The chosen method to reduce the particle size was sonication.

In the first experiment, the niosomal suspensions were centrifuged for 15 minutes at 4000 rpm, and 15 mL of the collected supernatant was further sonicated using a MS72 probe for 5 minutes with the instrument set at 50 % of I ts maximum power. The previously defined targeted specification for the average mean diameter was 200 nm, but the vesicles obtained with this sonication conditions presented higher diameters (254.6 nm ± 4.490%).

In a second experiment, 15 mL of the supernatant was sonicated with KE76 probe for 5 minutes with the instrument set at 50% of its maximum power. The particles prepared using this probe presented mean average diameters (224.6nm ± 2.108%) smaller than the ones developed using the probe MS72. Therefore, this KE76 probe was adequate to optimize

particle size. Therefore, satisfactory results have been obtained while employing these sonication conditions (same volume, probe and instrument power) but using 2 cycles of 5 minutes. Therefore, these sonication conditions were chosen to be used in all the experiments, and all the results were obtained using this method and conditions: centrifugation for 15 minutes at 4000 rpm, with 15 mL of the collected supernatant being further sonicated using the KE76 probe for 2 cycles of 5 minutes with the instrument set at 50% of its maximum power.

3.4. Characterization of niosomal suspensions

Colloidal suspensions were properly diluted with distilled water in order to avoid scattering phenomena, in a ratio of 1:9 (sample:water**).**

Particle size, polydispersion index (PDI), and the zeta potential of niosomes were measured by dynamic light scattering (DLS) using a Zetasizer Nanoseries ZS90 at an angle of 90 in 0.01 m with cells at 25ºC. The PDI was used as a measure of the average size distribution of the particles: PDI lower than 0.3 indicates an homogenous population for colloidal systems (87).

For particle size and zeta potential measurements, six independent samples were taken from each dispersion, and measured at 25±0.1°C. An average dimensional distribution was then calculated referring to the mode (*i.e*. the most frequently occurring value), which is the value that best matches the mean diameter of the vesicles.

4. Results and discussion

4.1. Selection of the production methods

From all the methods presented in the previous sections, the TLE-Paddle, TLE-Vortex and Reverse methods are the most used at a laboratorial scale, because of their simple procedure and common materials. These methods have also been widely reported in the literature, being thus selected to be used to produce the niosomal formulations during this research project.

TLE-Paddle and TLE-Vortex method are similar, but the first encompasses the agitation being performed by the paddles inserted in a mechanical stirrer, while the second includes a manual agitation, which may entail reproducibility errors between batches.

Even though the TLE-Paddle leads to more reproducible results than the TLE-Vortex, it has shown some difficulties with the repeatability of the positioning of the paddles, since these are not kept in the same angle and height during the formulation process.

The Reverse Phase method was selected among those described above as it is expected to lead to batches with reproducible major physical-chemical properties.

4.2. Selection of the reduction size method

Sonication and extrusion were the two procedures available to achieve niosome size reduction. The first one was selected as it has been described as leading to particles with lower mean average diameters (15,129).

4.3. Definition of the critical quality attributes (CQAs)

The first goals of our study were to select the composition ratio and the methodology most suitable to produce niosomes with the targeted specifications for size, PDI and surface charge.

The niosomal formulations will be administered through the intravenous route and therefore, those presenting mean average diameters higher than 200 nm will be excluded, because a diameter greater than 200 nm will activate the complement system and be quickly removed from the blood stream, accumulating in the liver and spleen. Nanoparticles must also be larger than 10 nm, to avoid renal filtration barrier. Nanoparticles 20 nm or less show the greatest tumor penetration, however it is difficult to obtain such a small diameter (130,131).

The PDI is acceptable for values under 0.3, however a good PDI must present a value under 0.2, and the smaller it is, the more homogenous is the suspension of the niosomes. The zeta

potential must present numbers lower than -30 mV or higher than +30 mV, to foresee repulsion in order to avoid aggregation and fusion phenomenon.

In summary, based on the above reasoning, we have considered the following thresholds as CQAs for the three assessed parameters: diameter under 200 nm, PDI under 0.3 and zeta potential ± 30mV.

The results presented bellow follow the work developed at Università degli Studi Firenze, Dept. Chemistry "Ugo Schiff", integrated into the Mura working group.

4.4. Niossomes' characterization results

The following three tables (Tables 7-9) contain all the data that have resulted from niosomes' characterization, namely using different composition ratios and methods to address their impact on the overall physicochemical properties of these carriers. Each table presents the results obtained for niosomes prepared employing the different methods, including average mean diameter, PDI and zeta potential, for six different ratio compositions.

As displayed in Table 7 below, niosomes prepared using the TLE-Paddle method presented mean average sizes ranging from 127.8 nm ± 0.907 (ratio 50:25:25) to 171.1 nm ± 4.067 (ratio 40:50:10). The overall average size of the niosomes was 150.55 nm, with a standard deviation of 15.82 nm. Regarding polydispersity index, niosomes prepared by TLE-Paddle presented PDI ranging from 0.159 ± 0.034 (ratio 50:25:25) to 0.215 ± 0.007 (ratio 35:40:25). Finally, the zeta potential results for the niosomes prepared by TLE-Paddle, ranging from 0.613 ± 1.5 and −28.3 ± 3.5 mV.

Table 7. Effect of different composition ratios on the particle size, polydispersity index (PDI) and zeta potential, of vesicles composed by Span60:CHL:Solulan produced by TLE-Paddle method, at 25ºC.

Mol%	Vesicle Size (nm ±S.D.)	PDI	Zeta Potential (mV)
50:40:10	170.3 ± 2.536	0.195 ± 0.009	-28.3± 3.50
40:50:10	171.2 ± 4.067	0.176 ± 0.011	-27.0 ± 1.80
35:40:25	144.5 ±1.401	0.215 ±0.007	-15.8 ± 1.720
50:25:25	127.8 ± 0.907	0.159 ± 0.025	0.613 ± 0.391

25:50:25	150.4 ± 3.394	0.192 ± 0.021	-15.1 ± 1.070
40:35:25	139.1 ± 1.258	0.187 ± 0.034	-17.6 ± 1.70

In Table 8 it can be shown that niosomes prepared using the TLE-Vortex method presented mean average sizes ranging from 136.0 nm ± 2,910 (ratio 35:40:25) to 257.9 nm ± 0,8505 (ratio 50:25:25). The vesicles with a diameter of 257.9 nm do not comply with the CQAs regarding the diameter. The overall average size of niosomes was 172.2 nm, with a standard deviation of 41.51 nm. Also, niosomes produced by TLE-Vortex method presented PDI ranging from 0.202 ± 0,8505 (ratio 35:40:25) to 0.389 ± 0.042 (ratio 50:25:25). The vesicles with a PDI of 0.389 PDI do not comply with the CQAs, because its value is higher than 0.3. Lastly, the zeta potential results for the niosomes prepared by TLE-Paddle method ranging from -15.7 ± 1.05 to -39.4 ± 2.29 mV.

The uncompliant results of diameter and PDI, regarding the characterization of the niosomes obtained by the ratio 50:25:25, leads us to consider them outliers or atypical values. These outlier results obtained for the ratio 50:25:25, are most likely the consequence of a laboratorial error that perpetuated itself by the incapability of a trial repetition. Nonetheless, there is a strong human handling component which might have, once again, compromised the trial.

Table 8. Effect of different composition ratios on the particle size, polydispersity index (PDI) and zeta potential, of vesicles composed by Span60:CHL:Solulan produced by TLE-Vortex method, at 25ºC.

Mol%	Vesicle Size (nm ±S.D.)	PDI	Zeta Potential (mV)
50:40:10	162.2 ± 3.109	0.209 ±.0.015	-37.6 ± 2.75
40:50:10	187.4 ± 1.054	0.290 ± 0.041	-39.4 ± 2.29
35:40:25	136.0 ± 2.910	0.202 ± 0.021	-15.7 ± 1.05
50:25:25	257.9 ± 0.8505	0.389 ± 0.042	-16.6 ± 1.23
25:50:25	149.8 ± 3.239	0.254 ± 0.019	-17.4 ± 1.16
40:35:25	143.0 ± 1.656	0.222 ± 0.014	-19.9 ± 1.63

Lastly, as observed in Table 9 below, niosomes prepared by the Reverse Phase method presented average mean diameters ranging from 123.9 nm ± 0.4583 (ratio 50:25:25) to 168.7 nm ± 2.499 (ratio 40:50:10). The average size of these niosomes is of 151.58 nm, with a standard deviation of 15.40 nm. Also, the niosomes prepared by this method presented PDI ranging from 0.186 ± 0.006 (ratio 50:25:25), to 0.223 ± 0.012 (ratio 25:50:25). Finally, the zeta potential results for the niosomes prepared by Reverse Phase method, ranging from -14.0 ± 1.10 to -28.3 ± 1.44 mV.

Table 9. Effect of different composition ratios on the particle size, polydispersity index (PDI) and zeta potential, of vesicles composed by Span60:CHL:Solulan produced by Reverse Phase method, at 25ºC.

Mol%	Vesicle Size (nm ±S.D.)	PDI	Zeta Potential (mV)
50:40:10	160.6 ± 1.514	0.215 ± 0.010	-25.7 ± 1.700
40:50:10	168.7 ± 2.499	0.217 ± 0.020	-28.3 ± 1.440
35:40:25	147.6 ± 1.400	0.221 ± 0.008	-16.6 ± 1.301
50:25:25	123.9 ± 0.4583	0.186 ± 0.006	-14.5 ± 0.777
25:50:25	165.3 ± 3.534	0.223 ± 0.012	-18.9 ± 1.18
40:35:25	142.8 ± 0.1155	0.194 ± 0.024	-14.0 ± 1.10

4.4. Influence of different production methods and molar composition ratios on niosomes particle size

We have analyzed the impact of the production methods (TLE-Paddle, TLE-Vortex and Reverse Phase method) and the different niosome composition on particle size (see Tables 7-9 in the previous section).

Comparing the three methods based on the previously referred parameters, we can conclude that the TLE-Vortex method led to niosomes with the highest average diameters.

According to our results the different composition ratios led to niosomes with different mean average sizes, despite the method employed for their preparation (Figure 9).

There is a similar profile of the bars, between the three methods. TLE-Paddle and Reverse Phase methods are the most similar ones - making it able to denote a pattern -since it presents a very different pattern of "particle size by composition ratios".

By comparing TLE-Paddle and Reverse phase methods, we can observe several similarities, namely the similar values for the average size of the niosomes produced (150.55nm and 151.58nm, respectively) and the relation between composition ratios and vesicles dimension: smaller niosomes were produced by the ratio 50:25:25 whereas larger niosomes were produced by the ratio 40:50:10 in both methods.

With this analysis it is possible to relate the amount of cholesterol in each formulation with the size of the particles. Formulations with less cholesterol (50:25:25 and 40:35:25) produce the smaller size niosomes, while formulations with more cholesterol produce the larger size niosomes. These relation between the amount of cholesterol and the size of the vesicle is supported in the existing bibliography, as presented in previous chapters.

Thus, we can conclude that 4/6 studied composition ratios (50:25:25, 40:35:25, 35:40:25, 40:50:10) influences the size of the vesicles, in a similar way in TLE-Pad and Reverse Phase methods. Accordingly, the niosomes presented similar mean average size despite the methodology used for their preparation, so we can say that the method of production do not have a great impact on the dimension of the produced vesicles.

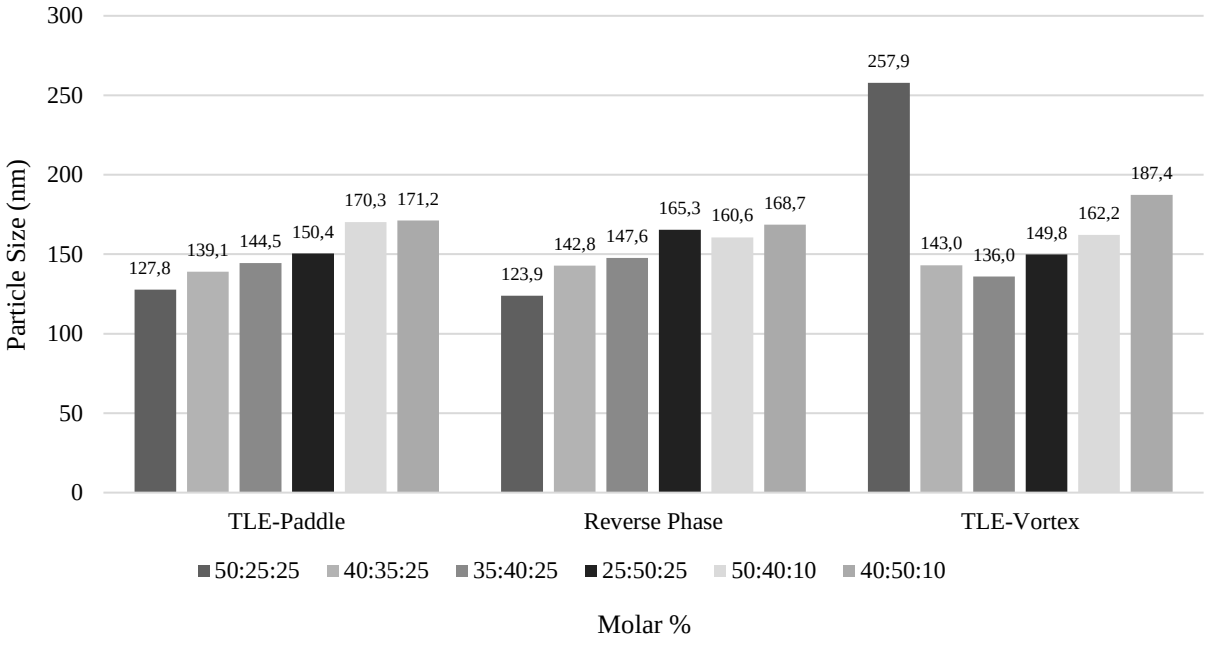

Figure 9. Variation of particle size of niosomes composed by different molar composition ratios (Molar%) and prepared by different methods (TLE-Paddle, TLE-Voxtex and Reverse Phase Method).

4.5. Influence of different production methods and molar composition ratios on niosomes PDI

The PDI is an indicator of the distribution of niosome size, that has the potential to be influenced by the production method used or the different composition ratios tested. The niosomes prepared by Reverse Phase method presented the lowest PDI (Figure 11). Nonetheless, the method that led to niosomes with the lowest PDI values was the TLE-Paddle method, since only one of the ratios tested resulted in PDI values superior to 0.2 (threshold to be considered for an optimal distribution of niosome size). As shown in figure 10, a homogeneous population was detected in our samples, with PDI values under 0.3, except for the identified outlier.

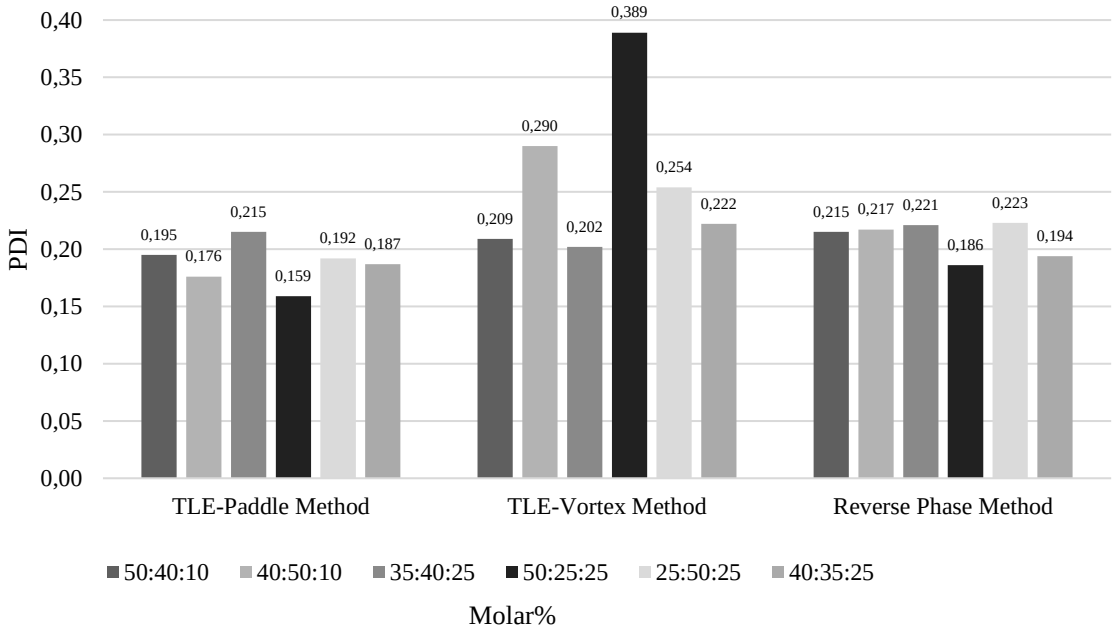

Figure 10. Variation of PDI of niosomes composed by different molar composition ratios (Molar%) and prepared by different methods (TLE-Paddle, TLE-Voxtex and Reverse Phase Method).

4.6. Influence of different production methods and molar composition ratios on niosomes zeta potential

The last parameter to be evaluated is the zeta potential that according to the definition for the selected critical quality attributes should be on the range of ±30mV.

Niosome zeta potential is considered a major physicochemical property to characterize the stability of colloidal systems. Higher zeta potential values anticipates vesicles repulsion, thus

overcoming system aggregation. Systems with the zeta potential value of ±30 mV or higher are considered to be stable (1,2,19,32,114).

According to the zeta potential data presented in Tables 7-9 previously analyzed, the majority of the niosomes that were produced, did not present an adequate electrostatic stability due to the inadequate zeta potential values.

Only the niosomes composed by 50:40:10 and 40:50:10 ratios and prepared by the TLE-Vortex method presented zeta potential values below -30mV (-37.6mV and -39.4mV values).

The obtained values for zeta potential do not reflect stable niosomes and the reasoning for this deserves some justifications.

First, the measurement of zeta potential is really sensitive, and may have been influenced by potential variations in two factors: room temperature and pH (132,133). Temperature is one of the factors that can have the greatest influence, however, the Zeta Sizer allows us to maintain a constant temperature, so this factor is not expected to have a great impact in the obtained results. Concerning potential variations in pH, these may have had lower influence in our zeta potential results, since pH of the Buffer were controlled daily.

A third factor that might have influenced the value of the zeta potential is the time between the end of the sonication (end phase of the productive process) and the measurement of the zeta potential. During the laboratorial work, it was clear that the zeta potential readings were affected by the time period ranging between the preparation of the niosomes and their characterization. It is important to understand the period needed to reach the stabilization of the system, prior to these measurements. Some of these formulations were characterized 30 minutes after their preparation, while others were stored overnight. However, due to the lack of time to complete our investigation, it was impossible to repeat the measurements in different time rest conditions.

The last topic to be discussed is related to the formulation of the niosomes.

The composition of the niosomal bilayer, and therefore the amount and type of the charge-inducing agents, affects the zeta potential of niosomes (32). The variability obtained for the zeta potential of nisomes indicates that a membrane bilayer is required to improve vesicle physical stability. Thus, we believe that adding a negatively charged ionic compound such

as dicetyl phosphate (DCP) to our noisome formulation, would improve our zeta potential results to values greater than -30mv.

Another important consideration is the morphological characterization of niossomes.

In order to improve our characterization of the empty niosomes, it would have been important to use the Transmission Electron Microscopy (TEM) technique. This type of analysis would have given us the information about the shape and the number of layers, making able also the evaluation of the existence of aggregation phenomenon (15). By using this TEM technique, it would have allowed us to study the impact of different production methods and composition ratios, on the morphology of niosomes (134).

4.7 Selection of the best molar compositon ratio for niosome preparation

In order to find the best method - according to our predefined criteria - to produce niosomes, we have developed a systematic analysis of our results (Table 10).

First, we have excluded zeta potential as a parameter of our analysis, due to the lack of proper results according to the previously presented reasons. Therefore, the analysis is based on the other two parameters: particle size and PDI.

In general, the TLE-vortex method presents the least relevant results. Therefore, this method has been excluded also from our analysis.

We have analyzed the results from TLE-Paddle and Reverse Phase methods and have selected the three best particle sizes and the three best PDI's for each method, ordering them by a color scheme: green for best results; yellow for second-best and red for the third best results. By pairing the best results for the two characteristics, we have constructed Table 10 that displays the best ratios compositions.

It is important to point out that there the impact of composition ratios and methods on niosome particle size is similar (lines 1 and 2, lines 3 and 4, lines 6 and 7), which is not observed for the PDI (Table 10).

We can consider that the 50:25:25 composition ratio is the best to produce the niosomes with the desired characteristics, and the 40:35:25 composition ratio is the second-best ratio, whatever method is used to produce niosomes (Table 10).

We believe that the results obtained are not consistent enough to select a third composition ratio, considering the particle size and PDI. However, the niosomes particle size results indicate that the 35:40:25 is the third best composition ratio.

Table 10. Mean average size and polydispersity index (PDI) obtained for best noisome formulations composed by Span®60:Colesterol:Solulan, and prepared by the TLE-Paddle or Reverse Phase methods.

Mol%	Preparation Method	Vesicle Size (nm ±S.D.)	PDI
50:25:25	TLE-Paddle	127.8 ± 0.907	0.159 ± 0.025
50:25:25	Reverse Phase	123.9 ± 0.4583	0.186 ± 0.006
40:35:25	Reverse Phase	142.8 ± 0.1155	0.194 ± 0.024
40:35:25	TLE-Paddle	139.1 ± 1.258	0.187 ± 0.034
40:50:10	TLE-Paddle	171.2 ± 4.067	0.176 ± 0.011
35:40:25	Reverse Phase	147.6 ± 1.400	0.221 ± 0.008
35:40:25	TLE-Paddle	144.5 ±1.401	0.215 ±0.007
50:40:10	Reverse Phase	160.6 ± 1.514	0.215 ± 0.010

Best Result Second-Best Result Third-Best Result

5. Conclusion

The development of novel drug delivery systems plays a leading role in pharmaceutical sector nowadays. Niosomes are drug delivery systems that can be utilized for controlled, sustained and targeted delivery of drugs. Interest in niosomes is increasing because of their ability to encapsulate both hydrophilic and hydrophobic drugs simultaneously. They also can be used to encapsulate drugs of natural origin, enzymes, peptides, genes and all varieties of drugs. Several studies have been performed with different types of niosomes for the delivery of vaccines, anticancer, anti-inflammatory and anti-infective agents, and other medicines.

Due to their flexibility in the route of administration, niosomes have been extensively studied for various applications, from topical, transdermal, oral to brain-targeted drug delivery.

They are easy to prepare at a low cost, in addition of allowing to achieve entrapment efficiencies higher than their analog system, liposomes. Relevant studies have demonstrated that niosomes improve the stability of the entrapped drug, reduce the dose required for the therapeutic effect, and enable the targeted delivery to a specific type of tissue.

Niosomes are promising delivery systems, and their potential can be further enhanced by novel preparation, loading and modification methods, as well as using alternative materials which allow great versatility. Additionally, controllable size and shape, routes of administration and better drug EE, will dictate the future for these delivery systems.

This versatile drug delivery system has great potential in the fields of pharmaceutical and cosmetic sciences, being promising tools for commercially available therapeutics.

Niosomes are one exciting sector, and plentiful therapeutic as well as commercial merits are provided by the incorporation of drug molecules in novel drug delivery systems. It is evident that new pathways have been paved and doors opened in the delivery of already existing as well as new drugs. However, some aspects including translation to clinical use, costly production and limited drug payload must be addressed in more extensive manner. From the manufacturing perspective, conventional methods have the merit of easy scale-up. Also, there is a need for new materials, for their quality of being biodegradable, biocompatible and low toxicity, that will be met in future and in combination with novel fabrication techniques.

Our study indicates that the compositions ratios (and not the production method) have the greatest impact on the niosomes' physicochemical properties. Also, we can consider that the

50:25:25 composition ratio led to niosomes meeting the targeted specifications, being followed by the 40:35:25 composition ratio.

Although it is still necessary to repeat the measurements of niosome zeta potential, these preliminary results are a good starting point to the next phase of this project, since we have already found the best possible options to continue the investigation in terms of production methods and composition rate.

In conclusion, there is still room for improvement, and tomorrow's drugs will be more challenging regarding drug delivery and the pharmaceutic science will have a really difficult but rewording task ahead.

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