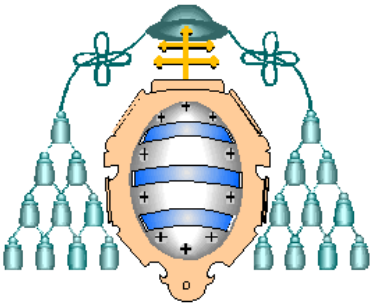
DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

UNIVERSIDAD DE OVIEDO



FORMULACIÓN Y PREPARACIÓN DE NIOSOMAS CONTENIENDO BIOCOMPUESTOS

TESIS DOCTORAL

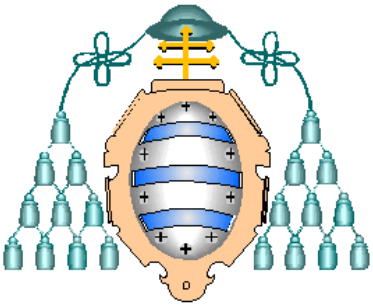
POR

DANIEL PANDO RODRÍGUEZ

NOVIEMBRE, 2014

DEPARTMENT OF CHEMICAL AND ENVIRONMENTAL ENGINEERING

UNIVERSITY OF OVIEDO



FORMULATION AND PREPARATION OF NIOSOMES CONTAINING BIOACTIVE COMPOUNDS

DOCTORAL THESIS

BY

DANIEL PANDO RODRÍGUEZ

NOVEMBER, 2014

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: FORMULACIÓN Y PREPARACIÓN DE NIOSOMAS CONTENIENDO BIOCOMPUESTOS	Inglés: FORMULATION AND PREPARATION OF NIOSOMES CONTAINING BIOACTIVE COMPOUNDS

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RESUMEN (en español)

El objetivo principal de esta Tesis Doctoral es la preparación de ***niosomas***, vesículas formadas por la agrupación de tensioactivos no iónicos de interés en la industria alimentaria, farmacéutica y cosmética, conteniendo ***trans*-resveratrol** (RSV), un compuesto biológicamente activo, con capacidad antioxidante y efectos beneficiosos para la salud de las personas.

Inicialmente, se estudió la viabilidad de los tensioactivos Span 80 y Span 60, junto con el colesterol como estabilizante de membrana, para formar niosomas conteniendo RSV. Estos sistemas niosomales se prepararon usando una técnica en dos etapas: agitación mecánica y sonicación. Las diferentes formulaciones se evaluaron mediante la determinación de su morfología, tamaño medio, distribución de tamaños, estabilidad y eficacia de encapsulación (EE) de los niosomas, junto con el análisis de la liberación *in vitro* del RSV. Cuando se utilizaban elevadas velocidades de agitación, los niosomas obtenidos presentaban un tamaño medio pequeño, una distribución de tamaños estrecha y una baja EE. Tanto el tamaño medio, como la estabilidad y la EE de los niosomas, se vieron afectados no sólo por la composición de los niosomas, sino también por el protocolo empleado en su preparación.

Teniendo en cuenta la importancia de las vesículas como sistemas de transporte y liberación controlada de principios activos, se prepararon niosomas y liposomas conteniendo RSV y se estudió la penetración de éste en piel de cerdo recién nacido utilizando celdas de difusión Franz. En este caso, también se usó colesterol como estabilizador de membrana y se estudió la influencia de la composición en las propiedades físico-químicas y estabilidad de estas vesículas. Para la formulación de los liposomas se utilizó fosfatidilcolina, mientras que para la preparación de los niosomas se utilizaron dos oleínas compatibles con la piel, de uso habitual como tensioactivos en formulaciones farmacéuticas. Los resultados obtenidos en este estudio mostraron que los niosomas son más adecuados que los liposomas para la

penetración y absorción percutánea del RSV.

Posteriormente, se llevó a cabo el nanodiseño de niosomas conteniendo RSV para administración oral utilizando tensioactivos de uso alimentario y dodecanol como estabilizante de membrana. Los niosomas se prepararon mediante el método de hidratación de película modificado y se estudió su viabilidad como aditivos en yogur. La selección del dodecanol como estabilizante de membrana tenía como finalidad evitar los posibles efectos adversos del colesterol en la salud humana, así como el rechazo de los consumidores de este alimento funcional. Para reducir el número de experimentos, se llevó a cabo un diseño factorial, así como un análisis de varianza (ANOVA) que permitió optimizar los parámetros más relevantes en la preparación de niosomas. La adición de niosomas conteniendo RSV no conllevó cambios en las propiedades texturales del yogur.

Finalmente, se prepararon niosomas para aplicación tópica utilizando dos procedimientos distintos: método de hidratación de película y sonicación (TFH-S) ligeramente modificado y método de inyección de etanol modificado (EIM). Los niosomas se formularon con Gelot 64 (G64) como tensioactivo y dos ácidos grasos muy frecuentes en formulaciones farmacológicas como potenciadores de la absorción percutánea: ácido oleico (OA) y ácido linoleico (LA). El método EIM resultó el más eficaz, ya que favorecía la obtención de niosomas con tamaños medios más pequeños, distribuciones de tamaño más estrechas y valores de EE de RSV más elevados que el método TFH-S.

RESUMEN (en Inglés)

The main objective of this thesis is the preparation of vesicles formed by the self-assembly of non-ionic surfactants (***niosomes***) of interest in food, pharmaceutical and cosmetic industries, containing *trans*-resveratrol (RSV), a bioactive compound with antioxidant capacity and beneficial effects for human health.

First, the suitability of niosomes to encapsulate RSV was studied using Span 80 and Span 60 as surfactants, and cholesterol as membrane stabilizer. Niosomes were prepared by a two-stage technique: mechanical agitation followed by sonication. The niosomal formulations were evaluated in terms of their morphology, mean size, size distribution, stability, entrapment efficiency (EE), and *in vitro* RSV release. High speed mechanical agitation produced niosomes with smaller mean size, narrower size distribution, and lower EE values. Mean size, stability and EE of the niosomes were affected not only by their composition, but also by the protocol used in their preparation.

Taking into consideration the importance of vesicular systems as nanocarriers in

dermal and transdermal delivery, RSV was incorporated into niosomes and liposomes and its ex-vivo percutaneous absorption in newborn pig skin was investigated using Franz diffusion cells. Cholesterol was also added as membrane stabilizer. The influence of vesicle composition on its physicochemical properties and stability was evaluated. Phosphatidylcholine was used in liposomes formulation while niosomes were formulated with two suitable skin-compatible oleins commonly used in pharmaceutical formulations as surfactants. The results obtained in this work enabled us to confirm that niosomes are more suitable than liposomes as carriers for percutaneous absorption of RSV.

Then, nanodesign of RSV entrapped niosomes for oral administration using food-grade surfactants and dodecanol as membrane stabilizer was carried out. RSV entrapped niosomes were prepared using a modified thin film hydration method, and their suitability as yoghurt additive was studied. Dodecanol was selected as a membrane stabilizer to avoid potential adverse health effects of cholesterol, as well as the rejection by the consumer of this functional food. A factorial design of experiments was carried out to reduce the number of experiments, and an analysis of variance (ANOVA) was used to optimize the key parameters involved in niosome preparation. The addition of niosomes containing RSV did not involve changes in the textural properties of regular yoghurt.

Finally, a new approach to formulate RSV entrapped niosomes for topical applications was proposed, and two specific preparation methods were applied and compared: thin film hydration and sonication method (TFH-S) with minor modifications, and ethanol injection modified method (EIM). Niosomes were formulated using Gelot 64 (G64) as surfactant and two suitable skin-compatible unsaturated fatty acids, commonly used in pharmaceutical formulations: oleic acid (OA) and linoleic acid (LA) as penetration enhancers. The EIM method allowed obtaining niosomes with smaller mean size, narrower size distribution and higher EE values than those provided by the TFH-S method.

AGRADECIMIENTOS

A la Prof. Carmen Pazos, Directora de esta Tesis, por permitirme formar parte del Grupo de Investigación de Emulsiones y Fenómenos Interfaciales, por sus consejos y apoyo en todo momento, por su enorme dedicación y paciencia a la hora de hacer correcciones y, sobretodo, por hacer de este pequeño grupo una gran familia.

Al Prof. José Coca, por permitirme formar parte del Grupo de Investigación de Emulsiones y Fenómenos Interfaciales, por sus enseñanzas y por ofrecerme siempre su colaboración y ayuda totalmente desinteresadas.

A la Dra. María Matos, Co-Directora de esta Tesis, por su gran amistad y ayuda durante todos estos años y por ser una compañera de trabajo excepcional.

Al Dr. José Manuel Benito, por estar siempre ahí cuando se necesita su colaboración.

Al resto de integrantes del grupo de investigación que han trabajo a mi lado: Dra. Gemma Gutiérrez, Dr. Miguel A. Suárez y David Allende, por su amistad y colaboración durante todos estos años.

A la Prof. Anna M. Fadda y a las Dras. Maria Manconi y Carla Caddeo de la Universidad de Cagliari (Italia), por hacer de mi estancia en su grupo una experiencia genial, por todo lo que me han enseñado, por su ayuda a pesar de estar a muchos kilómetros de distancia y por una perdurable amistad.

Al programa Severo Ochoa del Gobierno del Principado de Asturias, por la Beca Pre-Doctoral concedida para la realización de esta Tesis, así como al apoyo económico recibido del Gobierno de España a través del proyecto CTQ2010-20009-C02-01.

A todos los compañeros que trabajan y han trabajado en el este departamento, especialmente a Isma, Carlos, Rober y César, por todos los buenos momentos vividos y por su amistad.

A aquellas personas que han trabajado a mi lado durante un breve periodo de tiempo, porque su colaboración en esta Tesis ha sido de gran ayuda: Elena, Sonia, Miriam, Yarima, Ilay, Andrea y Rebeca.

A todo el personal de la facultad y en especial a Chema, Ángel, Tineo, Miguel, David y Bertín, por ese buen ambiente que generan a su alrededor.

Y, finalmente, a mi familia y a mi pareja por el apoyo recibido en todo momento.

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ABSTRACT

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1.INTRODUCTION

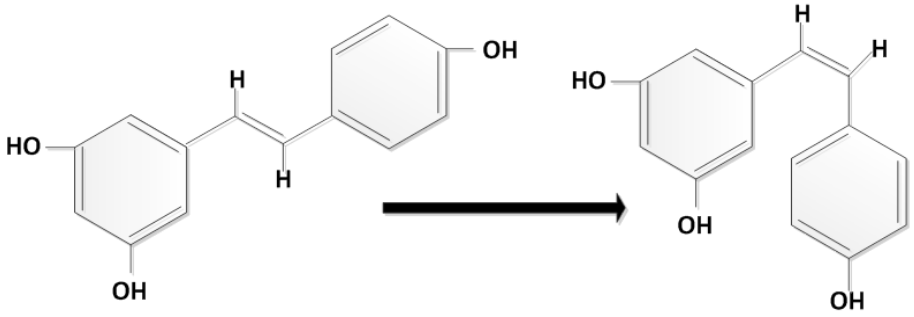
1.1. Resveratrol

Resveratrol (RSV) is a natural polyphenol from the stilbens family found in a wide variety of plants that can be considered an interesting compound to be used as *functional food* or *nutraceutical* ingredient, and also in topical use products because of its beneficial effects for human health.

RSV can be obtained either by extraction methods from plants or chemical synthesis. While the RSV used in food or cosmetic industries is commonly obtained from plants (Lantto*, et al.*, 2009; Soleas*, et al.*, 1997; Tomé-Carneiro*, et al.*, 2013), the pharmaceutical industry follows the organic synthesis path, where further research to obtain a more powerful biological activity is claimed (Delmas*, et al.*, 2011).

Although the molecule was first isolated in 1940, the main studies on RSV properties began in the nineties. Some of these benefits are anticancer activity (Karthikeyan*, et al.*, 2013; Surh*, et al.*, 1999), lifespan extension (Howitz*, et al.*, 2003), cardiovascular protection (Hung*, et al.*, 2000; Wu*, et al.*, 2013) antioxidant activity (Coradini*, et al.*, 2014; Frémont*, et al.*, 1999; Wu*, et al.*, 2013), protection to UV-B radiation (Kristl*, et al.*, 2009), inhibition of platelet aggregation (Magalhães*, et al.*, 2014), and anti-inflammatory activity (Magalhães*, et al.*, 2014; Ortega*, et al.*, 2012). Likewise, RSV seems to play an important role in the treatment and prevention of Alzheimer's disease (Björkhem*, et al.*, 2006; Dasgupta and Bandyopadhyay, 2013; Frozza*, et al.*, 2013; Lu*, et al.*, 2009).

It should be pointed out that most of the studies on RSV health effects were carried out *in vitro*. Some authors do not accept the equivalence *in vitro – in vivo* to RSV benefits due to its poor biodisponibilty, which is caused by its low stability, increased oxidation, and low solubility in water (Gambini*, et al.*, 2013; Ndiaye*, et al.*, 2011). In addition, RSV is a photosensitive molecule that exists as *cis* and *trans* structural isomers (Fig. 1), but only *trans*-RSV demonstrates the aforementioned health benefits. The exposure to light for 1 hour (Vian*, et al.*, 2005) leads to an irreversible change from the active *trans* isomer to the inactive *cis* one. For all these reasons, encapsulation of RSV seems to be a suitable approach to overcome these problems.



Trans-resveratrol *Cis*-resveratrol

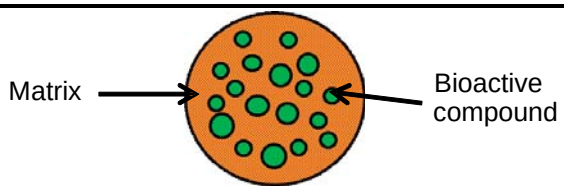
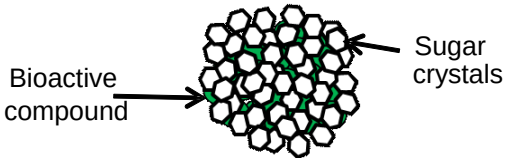
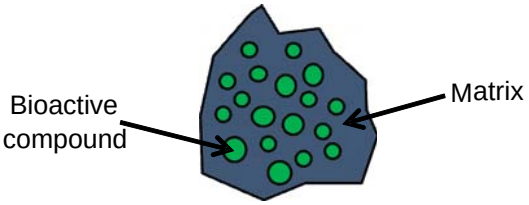
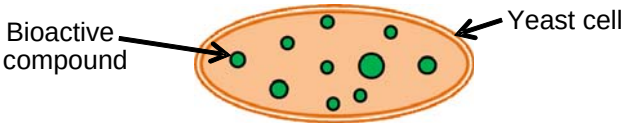
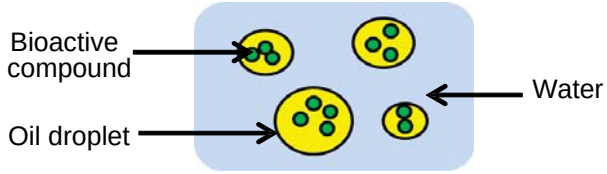
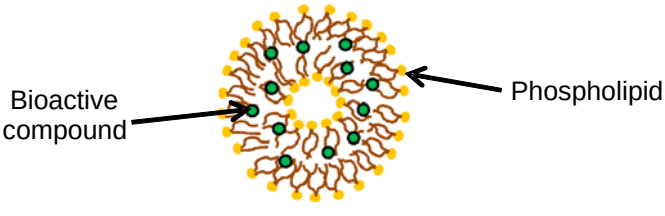
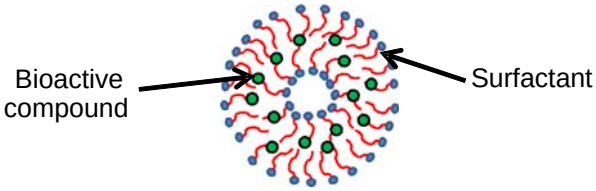
Fig. 1. Isomers of resveratrol

To exert a significant effect at cellular and molecular levels, RSV must penetrate into cells. Several works revealed a decrease of RSV accessibility to cells due to the numerous events at multiple stages implicated during the penetration process, mainly complexation with extracellular molecules, such as serum proteins, fatty acids, or lipoproteins. Thus, RSV encapsulation might be also useful to improve its penetration and delivery into cells (Delmas*, et al.*, 2011; Paolino*, et al.*, 2008).

1.2. Encapsulation methods

Encapsulation may be defined as a process to entrap one substance (active agent) within another substance (wall material) (Nedovic*, et al.*, 2011). Encapsulation studies have been carried out to protect RSV from degradation and to effectively mitigate its limitations. Several methods for the encapsulation of drugs or bioactive compounds have been reported, being the most common described in Table 1.

Table 1. Schematic illustration of the main encapsulation methods of drugs or bioactive compounds

Method	Illustration of characteristics
Spray drying	
Co-crystallization	
Freeze drying	
Yeast encapsulation	
Emulsification	
Vesicle entrapment	 

- ***Spray-drying.*** This encapsulation method has been used in the food industry since 1950. It is an economical, flexible, and continuous operation that allows entrapping a wide variety of compounds. Spray drying produces spherical particles with diameters ranging from 10 to 100 µm. The main limitation of this technique is the shortage of shell materials available to produce the particle matrix, since these materials must be soluble in water (Desai and Jin Park, 2005; Fang and Bhandari, 2010).

- ***Co-crystallization.*** It involves a modification of the crystalline structure of sucrose, from a perfect to an irregular agglomerated crystal, providing a porous matrix in which a second active compound can be incorporated. The spontaneous crystallization of sucrose is carried out at high temperatures (120 ºC) and the compound to encapsulate is added at the same time, which results in its incorporation into the void spaces inside the agglomerates of the microsized crystals, with a size less than 30 µm (Bhandari*, et al.*, 1998; Fang and Bhandari, 2010).

  The main advantages of this method are the high solubility, homogeneity and stability of the final encapsulated materials (Beristain*, et al.*, 1996).

- ***Freeze-drying.*** This is a technique mainly used to encapsulate heat-sensitive and water soluble compounds (Desai and Jin Park, 2005). It works by freezing the material and then reducing the pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase. The particles obtained have undefined forms and they are composed by a matrix containing the active compounds of interest (Fang and Bhandari, 2010).

- ***Yeast encapsulation.*** The structure of yeast cell wall and its natural properties made it an excellent encapsulation wall material with many benefits over other microencapsulation technologies (Nelson, 2002). Cells are grown in liquid culture medium. The encapsulation process involves mixing an aqueous suspension consisting of yeast cells and the compound to be encapsulated, which allows the compound to pass freely through the cell wall and the membrane, and to remain passively within the cell (Bishop*, et al.*, 1998).

  It has been reported that this method is suitable to entrap polyphenols, including RSV, although showing low entrapment efficiency values (~15%) (Shi*, et al.*, 2008).

- ***Emulsification.*** An emulsion is defined as a two-phase system consisting of two immiscible liquids of different composition, one in the shape of droplets, dispersed in the second one. Typically, the droplets diameter in food systems range from 0.1 to 100 µm.

Multiple emulsions are a special type of emulsion defined as ternary systems where the dispersed droplets contain smaller droplets of a different phase. They have either a water-in-oil-in-water ($W_1/O/W_2$) or an oil-in-water-in-oil ($O_1/W/O_2$) structure. The suitability of $W_1/O/W_2$ double emulsions to encapsulate *trans*-resveratrol with around 40% of entrapment efficiency has been reported (Hemar*, et al.*, 2010; Matos*, et al.*, 2014).

- ***Vesicle entrapment.*** Vesicles are colloidal particles in which a concentric bilayer made up of amphiphilic molecules surrounds an aqueous compartment. These vesicles are commonly used to encapsulate both hydrophilic and lipophilic compounds, for either food, pharmaceutical or cosmetic applications, and also can work as drug carriers. Hydrophilic compounds are entrapped into the aqueous compartments between the bilayers, while the lipophilic components are preferentially located inside the bilayer matrix (Saini*, et al.*, 2011; Uchegbu and Vyas, 1998). The most common types of vesicles are liposomes and niosomes.

  Liposomes were first described by Bangham and coworkers in 1965 (Bangham*, et al.*, 1965) and are, basically, spherical bilayer vesicles formed by the self-assembly of phospholipids. This process is based on the unfavorable interactions occurring between phospholipids and water molecules, where the polar head groups of phospholipids are exposed to the aqueous phases (inner and outer), and the hydrophobic hydrocarbon tails are forced to face each other in a bilayer (Da Silva Malheiros*, et al.*, 2010).

  Due to the presence of both lipid and aqueous phases in the structure of liposomes, they can be used for encapsulation, delivery, and controlled release of hydrophilic, lipophilic, and amphiphilic compounds (Da Silva Malheiros*, et al.*, 2010; Du Plessis*, et al.*, 1994).

  On the other hand, **niosomes** are vesicles formed from the self-assembly of non-ionic surfactants in aqueous media resulting in closed bilayer structures (Uchegbu and Vyas, 1998). As liposomes, their formation process is a consequence of the unfavorable interactions between surfactants and water molecules, and niosomes can also entrap hydrophilic, lipophilic, and amphiphilic compounds (Mahale*, et al.*, 2012; Moghassemi and Hadjizadeh, 2014). Niosome size range from 10 nm to 3 μm (Moghassemi and Hadjizadeh, 2014).

  The main advantages of niosomes with respect to liposomes (Marianecci*, et al.*, 2014; Uchegbu and Vyas, 1998) are the following:

  - Encapsulation of drugs or bioactive compounds into niosomes enhances its biodisponibilty
  - Niosomes have higher stability and lower cost than liposomes

- Niosomes encapsulation allows controlled drug delivery, since it is possible to modify the drug release rate and also to perform targeted drug delivery into specific locations.
- Niosomes are osmotically active, chemically stable, and have long storage time.
- Niosomes surface formation and modification are simple processes because of the functional groups on their hydrophilic heads.
- Niosomes are highly compatible with biological systems and have low toxicity because of their non-ionic nature.
- Niosomes are biodegradable and non-immunogenic.
- Niosomes can entrap both lipophilic and hydrophilic compounds
- Niosomes improve skin penetration of drugs or bioactive compounds.
- Variables involved in niosome formation can be easily controlled.
- Niosomes can enhance absorption of some drugs across cell membrane.
- Niosomes can be used for drug administration by different routes: transdermal, oral, pulmonary, ocular, parenteral, etc.

Since the aim of the present work is RSV encapsulation using niosomes, a more detailed section about this type of vesicles is included below.

1.3. Niosomes

Surfactants are amphiphilic molecules with two distinct regions consisting of a structural group, which has very little attraction for the solvent, and other group that has strong attraction for the solvent. The hydrophilic part is referred as the *head group* and the hydrophobic part as the *tail*. Depending on the nature of the hydrophilic group, surfactants can be classified as ionic or non-ionic. In the case of non-ionic surfactants, the surface-active portion has no apparent ionic charge.

As previously mentioned, niosomes are formed by the self-assembly of non-ionic surfactants resulting in closed bilayer structures (vesicles). The first report about non-ionic surfactant vesicles came from the cosmetic applications devised by L'Oreal, where it was reported that nonionic surfactants were preferred due to their less irritation power (Mahale*, et al.*, 2012).

As a result of this self-assembly, the hydrophilic head groups are oriented towards the aqueous medium as they enjoy maximum contact with it, whereas the hydrophobic parts of the surfactant molecule remain shielded from this aqueous medium. Fig. 2 shows the schematic structure of a niosome.

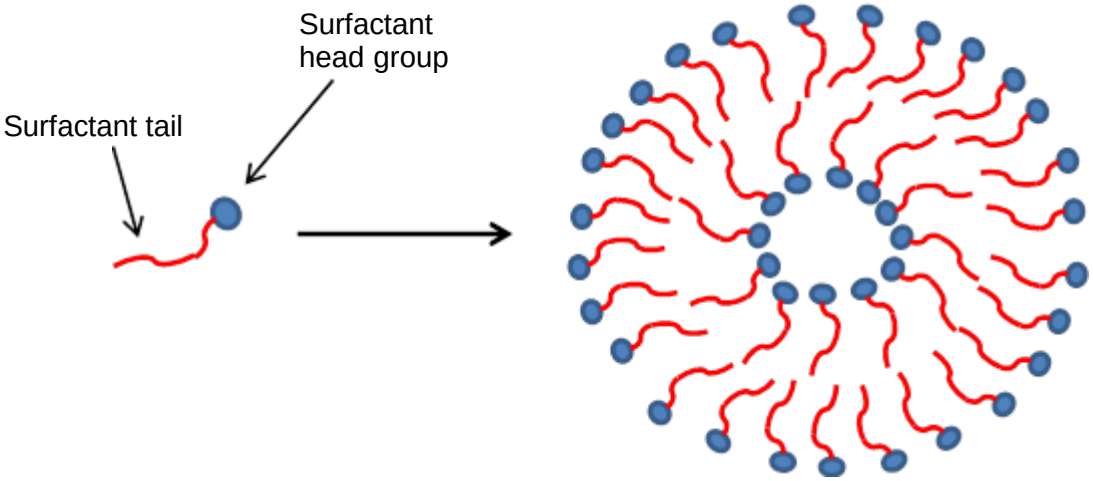


Fig. 2. Schematic structure of a niosome

1.3.1. FACTORS AFFECTING NIOSOMES FORMATION

The self-assembly of the surfactants into niosomes is governed by several key parameters (Uchegbu and Vyas, 1998), which are discussed below.

1.3.1.1. Formulation aspects

It is important to understand the basic components role in the niosomal structure before its preparation.

A. Non-ionic surfactant structure

The structure of non-ionic surfactants plays an important role in niosome formation. Normally, to form a bilayer membrane in aqueous media, a surfactant with higher contribution of the hydrophobic part is necessary. However, using an optimum level of hydrophobic membrane stabilizer (*e.g.* Cholesterol) niosomes can also be formed from hydrophilic surfactants (Manconi*, et al.*, 2006).

In order to select the appropriate surfactant, there are two key parameters to be considered: HLB and CPP.

The HLB (*Hydrophilic – Lipophilic Balance*) is a good indicator of the vesicle forming ability of a surfactant. This parameter is used on the basis that all surfactants combine hydrophilic and lipophilic groups in one molecule, and that the ratio between the weight percentages of these two groups for non-ionic surfactants is an indication of

the behavior that may be expected (Griffin, 1955). An HLB value of 1 corresponds to a completely lipophilic surfactant, and a value of 20 corresponds to a completely hydrophilic molecule. Table 2 shows the relationship between HLB range and surfactant application.

Table 2. HLB scale and surfactants application

HLB RANGE	APPLICATION
1-3	Antifoaming
3-6	Water-in-oil emulsions / Niosomes
7-9	Wetting and dispersion
8-18	Oil-in-water emulsions / Micelles
13-15	Detergency
15-18	Solubilization

The HLB values compatibles with niosomal formulation usually range between 4 – 8 (Saini*, et al.*, 2011), although it is also possible to obtain niosomes using surfactants with HLB values out of this range.

The CPP (*Critical Packing Parameter*) is also a useful parameter to establish the suitability of a surfactant to form niosomes. This parameter compares the contribution of hydrophobic and hydrophilic groups, and it is given by the following equation:

$$CPP = \frac{v}{l_c \, a_0} \quad (1)$$

Where v is the hydrophobic group volume, l_c is the critical hydrophobic group length, and a_0 is the area of the hydrophilic group head. Values of CPP between 1/2 and 1 indicate that the surfactant is suitable to form vesicles, values lower than 1/3 give spherical micelles, values from 1/3 to 1/2 form cylindrical micelles, values around 1 planar bilayers, and values higher than 1 inverted micelles (Israelachvili, 2011).

B. Additives

The use of additives in niosome formulation is very common to produce vesicles with high stability. Depending on where they exert their function, can be classified as follows:

- ***Membrane additives***. The most common is cholesterol, which is known to abolish the gel to liquid phase transition of liposomal and niosomal systems (Manosroi*, et al.*, 2003), as it changes the bilayer membrane fluidity and provides greater stability (Kumar and Rajeshwarrao, 2011). Cholesterol tends to improve the main vesicular properties, such as entrapment efficiency, release, and stability under storage (Biswal*, et al.*, 2008; Shilpa*, et al.*, 2011). It has also been recently reported that dodecanol could replace cholesterol as stabilizer in food-grade niosome formulations (Pando*, et al.*, 2015).

- ***Surface additives***. These compounds act on the niosome surface, being dicetyl phosphate (DCP) the most common one. DCP acts as a charge inducer usually imparting negative charge on the surface of niosomes, which remain stabilized by electrostatic repulsion (Waddad*, et al.*, 2013).

- ***Steric additives***. The most common is polyethylene glycol (PEG), which provides niosomes with steric repulsion and subsequent stabilization. The particular efficiency of surface-attached PEG chains has been explained by combination of their water solubility and flexibility, which sterically stabilize the vesicles, mainly preventing them from self-aggregation and/or fusion processes. (Garbuzenko*, et al.*, 2005; Lasic, 1994).

Fig. 3 shows the different types of stabilizers used in niosomes formulation.

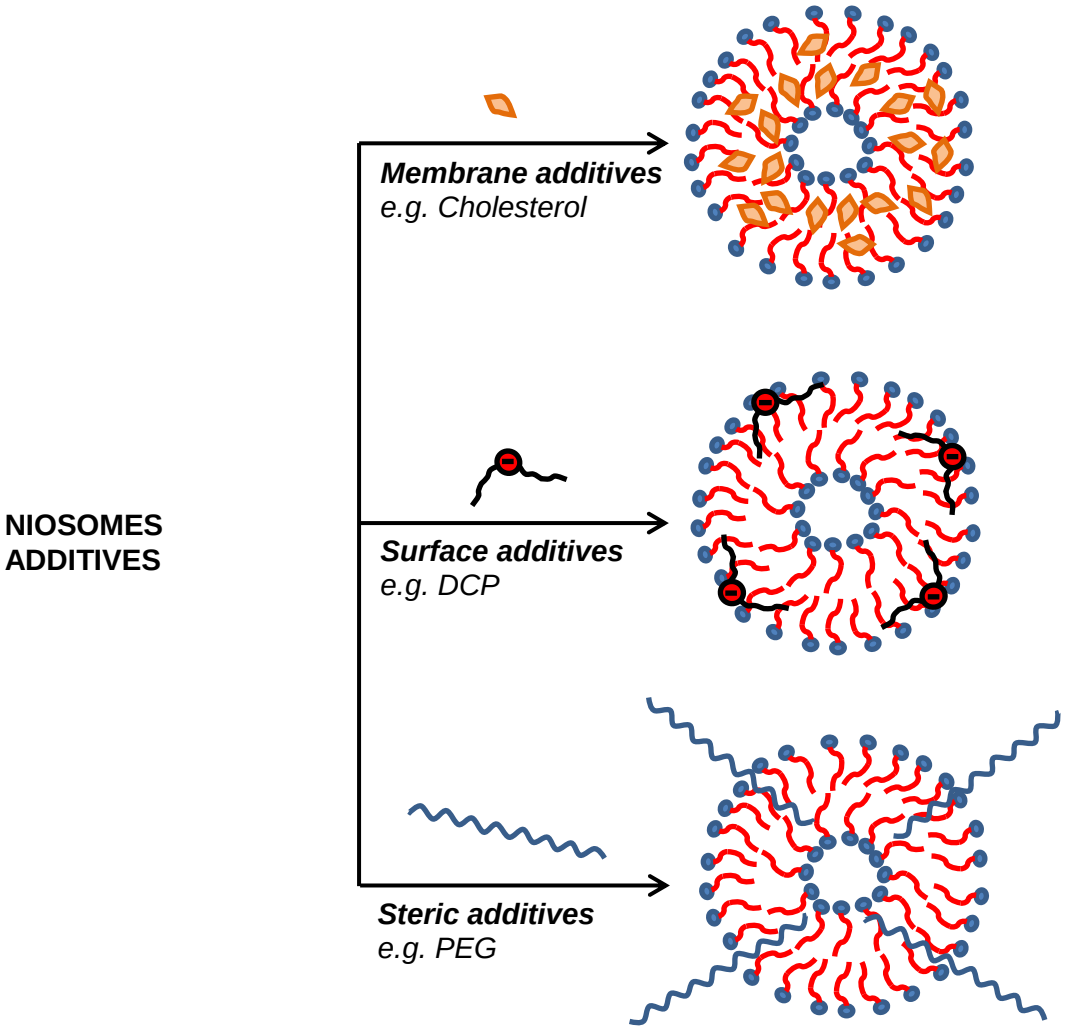


Fig. 3. Types of additives for niosomes

C. Encapsulated substance

The substances of interest, either drugs or bioactive compounds, to be incorporated into niosomes can also affect the morphology and stability of the final dispersion. However, it has been proved that amphiphilic compounds are the most problematic in this regard (Kumar and Rajeshwarrao, 2011; Uchegbu and Vyas, 1998). Doxorrubicin is an example of amphiphilic drug, and when it is encapsulated in niosomes, aggregation occurs and it should be overcome by the addition of a steric stabilizer (Kumar and Rajeshwarrao, 2011).

1.3.1.2. Surfactant concentration

The typical concentration of surfactant required to produce niosomes is generally 10–30 mM (1–2.5% w/w) (Lawrence*, et al.*, 1996; Uchegbu*, et al.*, 1992; Uchegbu*, et al.*, 1996; Zarif*, et al.*, 1994), although it is possible to produce niosomal dispersions

using concentrations out of this range. Normally, higher surfactant concentration involves higher drug entrapment efficiency, but it also leads to an increase in system viscosity (Pando*, et al.*, 2013a; Uchegbu and Vyas, 1998)

1.3.1.3. Temperature

The hydrating temperature commonly used to prepare niosomes should be above the gel to liquid phase transition temperature (Tc) of the system (Kumar and Rajeshwarrao, 2011; Marianecci*, et al.*, 2014). Niosomes prepared above Tc showed higher entrapment efficiencies that niosomes prepared below Tc (Biswal*, et al.*, 2008; Hao*, et al.*, 2002; Kumar and Rajeshwarrao, 2011).

1.3.2. NIOSOMES PREPARATION METHODS

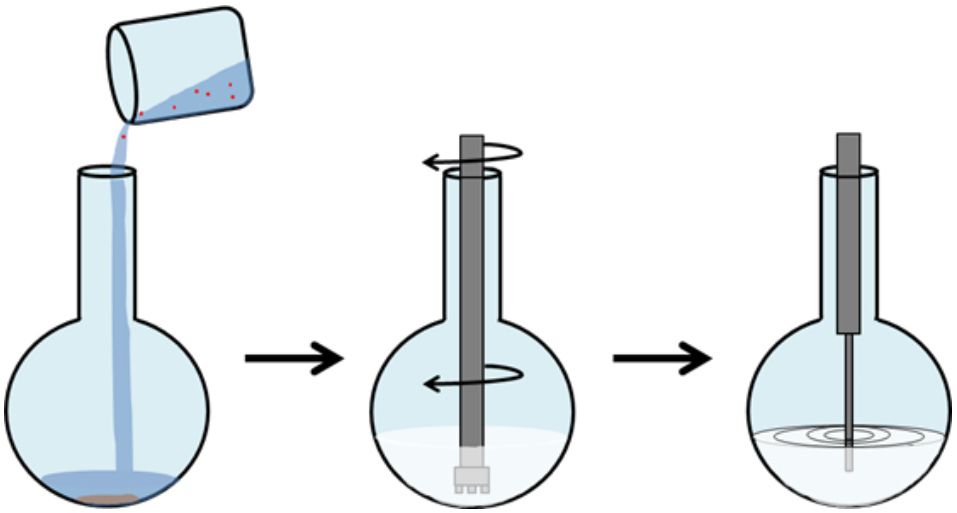
Niosomes formation is not a spontaneous process and normally requires an input of energy (Lasic, 1990). There are more than 10 different preparation methods reported in the literature (Moghassemi and Hadjizadeh, 2014; Walde and Ichikawa, 2001) although, in this section, only the most common are discussed.

Selection of the most appropriate method depends on surfactants and stabilizers present in the formulation, compound to be encapsulated, and characteristics required for the final application of niosomes.

1.3.2.1. Agitation – Sonication

In this method mechanical agitation (Liu and Guo, 2007), sonication (Pando, *et al.*, 2013b), or a combination of both (Pando, *et al.*, 2013a) are used as system energy input.

An aqueous solution containing the compound of interest is added to the surfactant/stabilizers mixture. Then, this mixture is stirred or sonicated to form niosomes. The best operational procedure seems to be an initial sample homogenization by agitation followed by sonication. Sonication is a technique broadly used to obtain small unilamellar vesicles (SUVs) with narrow size distribution. By contrast, other procedures, such as mechanical agitation, produce multilamellar vesicles (MLV) (Pando, *et al.*, 2013a; Walde and Ichikawa, 2001). A schematic diagram of this method is shown in Fig. 4.



Addition of an aqueous solution containing the bioactive compound to surfactant / stabilizers

Homogenization by agitation

Sonication

Fig. 4. Agitation – sonication method for niosomes preparation

1.3.2.2. Thin Film Hydration (TFH)

This method was first described by Bangham *et al.* (Bangham, *et al.*, 1965) and it is still widely used nowadays. TFH involves surfactant/stabilizers dissolution in an organic solvent, as chloroform or methanol, followed by organic solvent removing by vacuum evaporation, using a rotary evaporator. A thin film is formed on the wall of the flask, which is then hydrated with an aqueous solution containing the substance of interest. (Hao and Li, 2011; Manconi, *et al.*, 2006).

Depending on the final application of formulated niosomes, different homogenization techniques can be applied to the aqueous solution from hydration process: manual shaking or agitation lead to MLV, while sonication leads SUV niosomes. In general, this method provides good niosomes dispersion (Flower and Perrie, 2013).

A schematic diagram of this procedure is shown in Fig. 5.

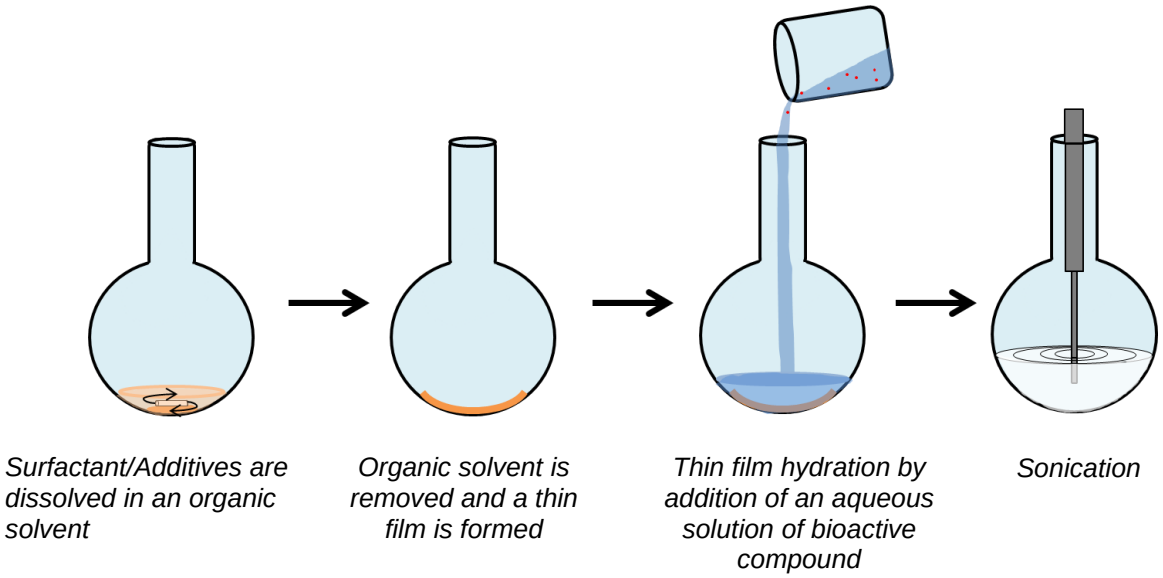


Fig. 5. Thin Film Hydration method for niosomes preparation

1.3.2.3. Dehydration – Rehydration Vesicles (DRV) method

This method was first described by Kirby and Gregoriadis (Kirby and Gregoriadis, 1984). Application of this procedure usually involves a first stage where niosomes are obtained by TFH method. Then, they are frozen in liquid nitrogen and freeze-drying overnight. Finally, the dried cake formed is hydrated and sonicated again to form the final niosomes.

This method produces niosomes with high mean size and good entrapment efficiency (Kawano, *et al.*, 2003; Mugabe, *et al.*, 2006). Fig. 6 shows a schematic diagram of this method.

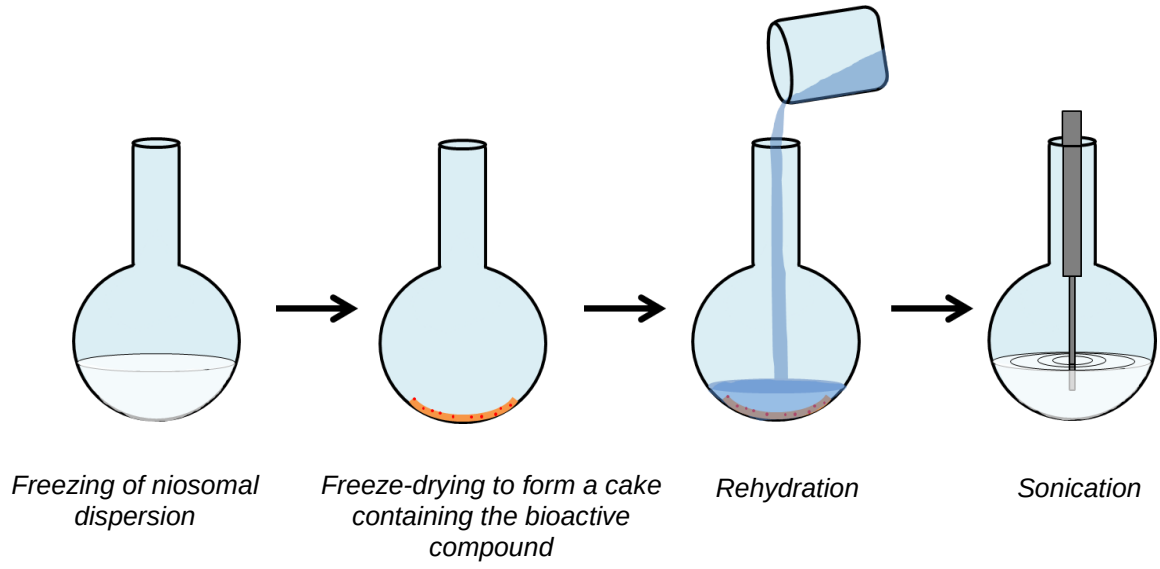


Fig. 6. Dehydration – Rehydration Vesicles method for niosomes preparation

1.3.2.4. Reverse Phase Evaporation (REV)

In this method, surfactant and additives are previously dissolved in an organic solvent. Then, the organic solution is added to an aqueous phase, containing the compound of interest, and this mixture is sonicated to obtain an emulsion. Finally, the organic solvent is slowly removed under vacuum evaporation until hydration is completed and niosomes are formed (Abdelkader*, et al.*, 2011; Guinedi*, et al.*, 2005). This procedure enables to obtain large unilamellar vesicles (LUV) (Moghassemi and Hadjizadeh, 2014). Fig. 7 shows a schematic diagram of this method.

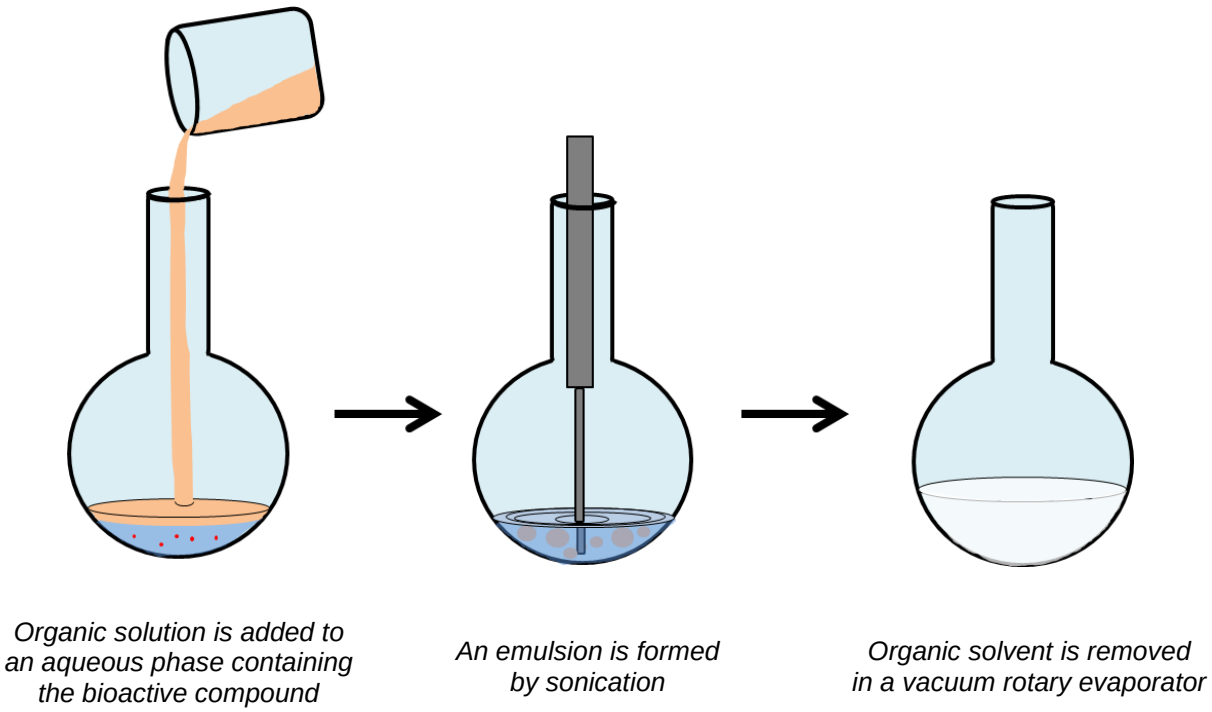


Fig. 7. Reverse Phase Evaporation method for niosomes preparation

1.3.2.5. Ether Injection Method (EIM)

In this technique, surfactant and additives are first dissolved in an organic solvent, as diethyl ether or ethanol, and then injected slowly through a needle into an aqueous phase containing the bioactive compound. Then, the organic solvent is removed using a vacuum rotary evaporator. When ethanol is used as organic solvent, niosomes are formed during the injection process, while niosomes are formed after organic solvent is removed if diethyl ether injection is used (Wagner*, et al.*, 2002). Depending on experimental conditions, vesicles size range between 50-1000 nm. This method can be easily scaled-up (Wagner*, et al.*, 2002). A schematic diagram of this procedure is shown in Fig. 8.

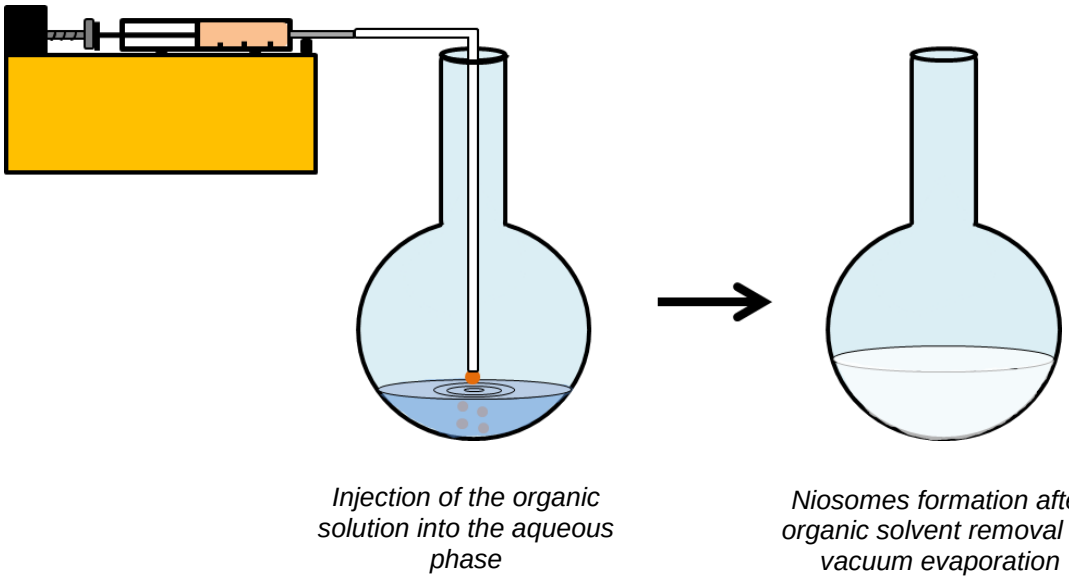


Fig. 8. Ether Injection Method for niosomes preparation

1.3.3. NIOSOMES CHARACTERIZATION

The most important parameters to characterize niosomes are the following:

1.3.3.1. Vesicle mean size and size distribution

Niosomes are assumed to be spherical in shape, therefore their size is given as a mean value (Z-Average), which can range from 20 nm to 50 µm (Moghassemi and Hadjizadeh, 2014). Polydispersity Index (PDI or PI) is the parameter used to characterize the distribution of niosomes size (Pando, *et al.*, 2013b). A PDI value of 0 indicates completely monodispersed vesicles, while a value of 1 implies highly polydispersed vesicles. In this study, both the mean (Z-Average) size and PDI of niosomes were determined via Dynamic Light Scattering (DSL) using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK).

1.3.3.2. Zeta potential (ζ-potential)

This parameter represents the electric potential at the plane of shear, located approximately between the compact and diffuse layers. In other words, ζ-potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle (Clogston and Patri, 2011). The ζ-potential is a good indicator of the stability of niosomes. In general, high absolute values indicate electrostatic repulsion between vesicles and are linked to high stability, since it prevents aggregation and fusion of niosomes (Pando, *et al.*, 2013b).

1.3.3.3. Stability

The main problems associated with vesicles storage are aggregation, fusion, and leakage of the compound encapsulated. Therefore, in this work the stability of niosomes was monitored with time by measuring backscattering (BS) profiles in a Turbiscan Lab® Expert apparatus (Formulaction, France) with an Ageing Station incorporated (Formulaction, France). These profiles show a macroscopic fingerprint of the niosomes at a given time, providing useful information about changes in vesicles size distribution, and/or appearance of a creaming layer or a clarification front with time (Pando, *et al.*, 2013a).

1.3.3.4. Morphology

To obtain information about the niosomal structure, as well as to confirm vesicles morphology, analysis of niosomes was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM (Japan).

1.3.3.5. Entrapment efficiency

It is usually determined by measuring the difference between entrapped and total amount of drug or bioactive compound initially incorporated. Different analytical techniques must be used depending on compound encapsulated. In this work, RSV was determined by chromatography (HP series 1100 chromatograph, Hewlett Packard, USA) using UV/VIS absorbance and fluorescence detectors.

Table 3 summarizes the different techniques used to characterize niosomes.

Table3. Methods used to characterize niosomes

Parameter	Method
Average size	Dynamic Light Scattering (DLS) Transmission Electron Microscopy (TEM)
Size distribution	DLS
ζ-potential	Phase Analysis Light Scattering (PALS)
Stability	Multiple Light Scattering (MLS)
Morphology	TEM
Entrapment efficiency	High Performance Liquid Chromatography (HPLC) Spectrophotometry

1.3.4. APPLICATIONS

Niosomes were first used in the cosmetic industry and have also gained attention in pharmaceutical companies due to their high efficiency to entrap different types of drugs or bioactive compounds (Shilpa*, et al.*, 2011). For this reason, and taking into account the aforementioned advantages, niosomes can be used as nanocarries in several delivery systems, such as: antioxidant, anticancer, anti-inflammatory, anti-asthma, antimicrobial, anti-amyloid, anti-Alzheimer, anti-bacterial, anti-malarial, anti-fungal, gene delivery, nutraceuticals, etc. (Moghassemi and Hadjizadeh, 2014).

1.3.4.1. Dermal and transdermal delivery

Dermal delivery consists on the topical application of a drug or bioactive compound into the skin either for treatment of diseases or to obtain health benefits. Niosomes can increase the residence time of the drug in skin deeper layers, as epidermis and dermis, allowing to obtain higher concentrations at specific locations or sites of action, reducing the systemic absorption of the drug, and increasing the treatment efficiency (Manconi*, et al.*, 2006; Marianecci*, et al.*, 2014; Pando, Daniel*, et al.*, 2013).

Further, transdermal delivery is used as an alternative route of drug administration, instead of oral or parental routes. This drug delivery route shows several advantages, such as avoidance of risks and inconveniences of intravenous

therapy, avoidance of first pass hepatic metabolism, no gastrointestinal degradation, and avoidance of several issues due to oral administration (*e.g.*: vomiting) (Marianecci, *et al.*, 2014)

Fig. 9 shows dermal and transdermal delivery of the drug into the skin.

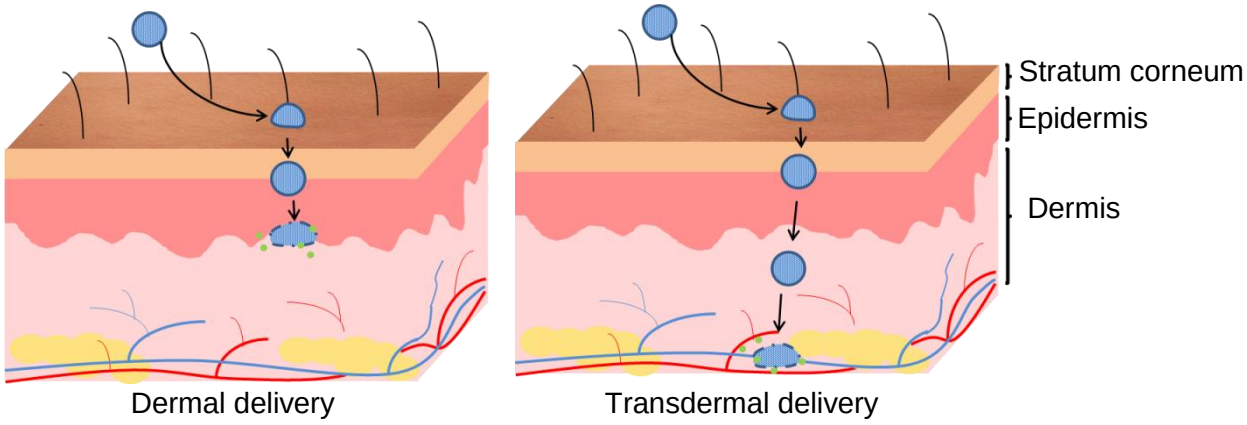


Fig. 9. Dermal and transdermal delivery using niosomes

1.3.4.2. Oral delivery

Oral is one of the most common routes of drugs administration. In addition, nowadays the use of functional foods and nutraceutics is increasing due to the health benefits provide by these products (Shahidi, 2009).

Compounds administrated orally can present bioavailability problems due to several reasons, such as: poor solubility, low dissolution rate, degradation, and unpredictable absorption. Niosomes encapsulation can protect the entrapped compound increasing its bioavailability (Gurrapu, *et al.*, 2012; Pando, *et al.*, 2013a) and also help to mask undesirable flavors (Tavano, *et al.*, 2014).

1.3.4.3. Pulmonary delivery

In inflammatory diseases, such as infections or cancer of the respiratory tract, pulmonary delivery is an interesting route of administration, instead of oral or parenteral ones, due to high drug concentrations located at the specific site of action. On the other hand, some lipophilic drugs do not easily permeate through the hydrophilic mucus to reach this site of action. For this reason, targeted delivery using niosomes helps to carry the drug to specific location amplifying its therapeutic effect (Marianecci, *et al.*, 2014; Terzano, *et al.*, 2005).

Among different aerosol delivery technologies, nebulizers seem to be simple and adequate devices, because vesicular structures may be delivered without further processing (Saari*, et al.*, 1999).

1.3.4.4. Ocular delivery

In topical ocular drug delivery there are many anatomical and physiological barriers to overcome, and a good strategy is the use of carriers (Marianecci*, et al.*, 2014). Several authors have confirmed that controlled delivery of some ophthalmic drugs improves their ocular bioavailability with respect to traditional eye drops (Abdelbary and El-Gendy, 2008; Di Colo and Zambito, 2002).

The advantage of using niosomal systems as ophthalmic nanocarriers does not only reside in providing prolonged and controlled delivery at the corneal surface, but also in prevent drug metabolism from the enzymes present at the tear/corneal epithelial surface (Abdelbary and El-Gendy, 2008).

1.3.4.5. Parenteral delivery

Parenteral route is one of the most common and effective routes of administration for drugs or bioactive compounds. Normally, parenteral route requires frequent injections to maintain an effective concentration of the therapeutic drug. One of the major progresses in the field of drug delivery system was the development of vesicles capable to provide targeted and sustained drug release in predictable manner to overcome the problems associated with conventional parenteral delivery systems (Marianecci*, et al.*, 2014).

Nowadays, niosomes containing active drugs for parental administration are developed for several diseases treatment, such as melanoma, fungal and viral diseases, etc. These niosomes show high efficiencies compared to conventional treatments (Manosroi*, et al.*, 2013; Shi*, et al.*, 2006; Wang*, et al.*, 2012).

1.3.4.6. Gene delivery

Gene therapies, using genetic material (DNA, oligonucleotides, small interfering RNAs, ribozymes, DNAzymes), rather than traditional drugs, are being investigated for treatment of different inherited or acquired disorders. Gene therapy has the theoretical potential to treat almost any disease (Marianecci*, et al.*, 2014). Niosomes seem to be a great tool for topical gene delivery, since topical immunization using niosomes has received a great deal of interest due to its painless and easy administration (Mahale*, et al.*, 2012).

1.3.4.7. Therapeutics / Diagnostics

The use of vesicles as nanocarriers has also emerged as a successful strategy to enhance targeted delivery into specific cells using antibody/antigen recognition (Simard and Leroux, 2009), In the last 15 years, this technology (mainly focused on liposomes) has matured and now several vaccines containing vesicles-based adjuvants have been approved for human use, or have reached late stages of clinical evaluation (Watson*, et al.*, 2012). It is important to notice that niosomes can be used for these purposes. In addition, niosomes can also be used as imaging agents for tumors or for making biosensors to detect diseases, increasing the potential for diagnosis at an earlier stage (Uchegbu and Vyas, 1998; Yu*, et al.*, 2011).

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2.OBJECTIVES

In this work, the following specific objectives have been pursued:

- Preparation of food-grade resveratrol (RSV) entrapped niosomes by a two-stage technique: mechanical agitation and sonication. Determination of the operation parameters influence on mean size and vesicle size distribution, stability, entrapment efficiency, and *in vitro* RSV release of niosomes.

- Encapsulation of RSV into niosomes and liposomes for topical use. Study and comparison of ex-vivo RSV percutaneous absorption on new born pig skin using Franz diffusion cells. Study of composition effect on physicochemical properties and stability of these vesicles.

- Nanodesign of RSV entrapped niosomes for oral administration using food-grade surfactants and dodecanol as membrane stabilizer. Evaluation of their feasibility as yoghurt additives. Application of an analysis of variance (ANOVA) to optimize the key parameters involved in niosome preparation.

- Formulation of RSV entrapped niosomes for topical delivery using two fatty acids as penetration enhancers. Comparison of two different preparation methods: *thin film hydration and sonication method* (TFH-S) with minor modifications, and *ethanol injection modified method* (EIM). Development of an ex-vivo transdermal study on new born pig skin using Franz diffusion cells.

3.RESULTS

I. Preparation and characterization of niosomes containing resveratrol

Polyphenols are a typical example of antioxidants of food interest. This is the case of resveratrol (RSV), a phytoalexin found in red wine and a wide variety of fruits and edible plants. RSV has beneficial effects for human health, because of its antioxidant, anti-inflammatory, cardioprotective and anti-tumour activity. Therefore, it can be considered as a novel *nutraceutical* and be used as an ingredient in functional food. However, its application is compromised by its low water solubility, short biological half-life, rapid metabolism and fast elimination. RSV is an easily oxidizable and extremely photosensitive compound, and its exposure to light leads to an irreversible change from the active *trans* isomer to the inactive *cis* isomer. Hence, appropriate protection of RSV is required.

The goal of this work was to investigate the suitability of niosomes to encapsulate RSV. Niosomes were formulated with Span 80 or Span 60 using cholesterol as membrane stabilizer. They were prepared by a two-stage technique: mechanical agitation and sonication. The characterization of niosomal formulations was carried out measuring the following parameters: morphology, mean size and particle size distribution, stability, entrapment efficiency (EE) and *in vitro* RSV release.

Article 1. D. Pando, G. Gutiérrez, J. Coca, C. Pazos. *Preparation and characterization of niosomes containing resveratrol*.

Journal of Food Engineering 117 (2013) 227-234 DOI: 10.1016/j.jfoodeng.2013.02.020

Personal contribution to work

In this study I gathered information regarding the state-of-the-art about RSV vesicle entrapment and the different preparation methods for these systems reported in the literature. I conducted the experiments and prepared the first manuscript draft, with the advice of Prof José Coca, and under the supervision of Prof. Carmen Pazos. RSV release calculations and Figures attached were performed in cooperation with Dr. Gemma Gutiérrez.

I. Preparación y caracterización de niosomas conteniendo resveratrol

Los polifenoles son un ejemplo característico de antioxidantes de interés alimentario. Este es el caso del resveratrol (RSV), una fitoalexina presente en el vino tinto y en una gran variedad de frutos y plantas comestibles. El RSV posee efectos beneficiosos para la salud humana debido a sus propiedades antioxidantes, antiinflamatorias, cardioprotectoras y antitumorales. Por lo tanto, el RSV se puede considerar como un *nutracéutico* novedoso y usarse como ingrediente en alimentos funcionales. Sin embargo, sus aplicaciones se encuentran limitadas como resultado de su baja solubilidad en agua, corta vida media biológica, acelerado metabolismo y rápida eliminación, así como de su facilidad de oxidación y elevada fotosensibilidad. En este sentido, la exposición a la luz conduce a un cambio irreversible del isómero activo *trans* al isómero inactivo *cis*, lo que justifica la evidente necesidad de proteger al RSV de manera adecuada.

El objetivo de este trabajo ha sido investigar la viabilidad de los niosomas para encapsular y proteger al RSV. Los niosomas se formularon con Span 80 o Span 60, usando colesterol como estabilizante de membrana. Para su preparación se siguió una técnica en dos pasos: agitación mecánica y sonicación. La caracterización de las formulaciones niosomales se llevó a cabo determinando los siguientes parámetros: morfología, tamaño medio y distribución de tamaños, estabilidad, así como eficacia de encapsulación (EE) y liberación *in vitro* del RSV.

Artículo 1. D. Pando, G. Gutiérrez, J. Coca, C. Pazos. *Preparation and characterization of niosomes containing resveratrol*

Journal of Food Engineering 117 (2013) 227-234. DOI: 10.1016/j.jfoodeng.2013.02.020

Contribución personal al trabajo

En este estudio, recopilé información acerca del estado del arte sobre la encapsulación de RSV en vesículas, así como sobre los diferentes métodos de preparación de estos sistemas descritos en la bibliografía. Asimismo, llevé a cabo los experimentos y redacté el borrador del artículo, con el asesoramiento del Prof. José Coca y la supervisión de mi directora de Tesis Prof. Carmen Pazos. Para los cálculos de la liberación del RSV y la elaboración de las figuras, conté con la colaboración de la Dra. Gemma Gutiérrez.

Journal of Food Engineering 117 (2013) 227–234

Contents lists available at SciVerse ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

Preparation and characterization of niosomes containing resveratrol

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ARTICLE INFO

Article history:
Received 18 October 2012
Received in revised form 19 February 2013
Accepted 20 February 2013
Available online 1 March 2013

Keywords:
Resveratrol
Niosomes
Span 80
Span 60
Release
Entrapment efficiency

ABSTRACT

Resveratrol (RSV) is an antioxidant present in red wine that may be used in functional foods. Niosomes capable on entrapping and retaining RSV were prepared in Span 80 and Span 60-cholesterol (weight ratio 1:1) systems by a two-stage technique: mechanical agitation and sonication. The niosomal formulations were evaluated for morphology and particle size, stability, entrapment efficiency and *in vitro* RSV release. Niosomes made of Span 80 were very stable but had low entrapment efficiency, while niosomes made of Span 60-cholesterol had better entrapment efficiency but a less monodisperse size distribution. Higher entrapment efficiencies were obtained when low agitation was used in the first stage of the preparation procedure.

Release studies showed that niosomes made of Span 60-cholesterol release RSV at a slower rate than niosomes made of Span 80.

1. Introduction

Niosomes (non-ionic surfactant vesicles) are microscopic vesicles consisting of an aqueous core enclosed by a membrane of non-ionic surfactants that form closed bilayer structures based on their amphiphilic nature (Uchegbu and Vyas, 1998). The lipophilic groups are located in the interior of membrane while the hydrophilic groups are exposed to the aqueous medium. It is shown that in general niosome and liposome structures do not form spontaneously (Lasic, 1990; Sahin, 2007) and they need some energy input (e.g., physical agitation or heat), which leads to different preparation techniques. The vesicles entrap the active compound/drug that can be released at a controlled rate.

Niosomes can be prepared by hydration of the surfactant to obtain a colloidal dispersion that entraps the desired compound. The compound that remains in the hydrating solution is separated from the vesicles by gel chromatography (e.g. Sephadex G50), centrifugation or dialysis (Uchegbu and Vyas, 1998). The properties of the vesicle membrane are influenced by the method of preparation. The most common methods are: sonication (used in this work) (Hua and Liu, 2007), thin film hydration (Hao and Li, 2010) and reverse-phase evaporation (Guinedi et al., 2005). However, the last two techniques involve the use of organic solvents, and may not be appropriate for some applications of niosomes. Formulations of niosomes were first developed by L'Oreal cosmetic laboratories (Handjani-Vila et al., 1979) but they have found applications in many areas as drug targeting agents. They have been also used in food applications as they can encapsulate both hydrophilic and lipophilic components: hydrophilic components in the aqueous layer and lipophilic components by attachment to the vesicular membrane (Carafa et al., 1998). In all instances the surfactants should be biocompatible and exhibit negligible toxicity. Niosomes and liposomes are both employed in producing cosmetic commercial market, but niosomes offer two main advantages: their higher chemical stability and the lower cost (Uchegbu and Vyas, 1998). The inclusion of cholesterol as stabilizer increases the hydrodynamic diameter and entrapment efficiency. This approach has been successfully used by Manosroi et al. (2003).

Resveratrol (RSV) is a natural polyphenol produced by plants in response to abiotic stresses, such as exposure to ultraviolet (UV) light or the presence of heavy metal ions. RSV is found primarily in the seeds and skin grapes and red wine, but it is also present in nuts and blackberries. RSV can be considered as a novel *nutraceutical* and to use it as an ingredient of functional foods could provide consumers with a wide range of benefits.

RSV shows geometric isomerism, but only trans-resveratrol (trans-3,4,5-trihidroxystilbene) appears to have biological effects such as anticancer activity (Jang et al., 1997; Surth et al., 1999), lifespan extension (Howitz et al., 2003), cardioprotection (Hung et al., 2000), antioxidant activity (Frankel et al., 1993; Fremont et al., 1999; Siemann and Creasy, 1992), protection to UV-B radiation (Kristl et al., 2009) inhibition of platelet aggregation (Bertelli et al., 1995; Chung et al., 1992) and antiinflammatory activity (Pace-Asciak et al., 1995). In recent years investigations

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http://dx.doi.org/10.1016/j.jfoodeng.2013.02.020

of the use of cell protection for using RSV from Aβ-induced oxidative stress seems to play an important role in the Alzheimer's disease process (Lu et al., 2009; Han et al., 2004; Jang and Surh, 2003; Russo et al., 2003; Savaskan et al., 2003; Sharma and Gupta, 2002).

Microencapsulation technology has been widely used to protect core materials in the field of food (Bustos et al., 2003; Chen et al., 2006). RSV is a photosensitive molecule, and exposure to light leads to an irreversible change from the *trans* to the inactive *cis* isomer, hence, appropriate protection of RSV is required. Moreover, when RSV is used in drug delivery, cosmetics or as a food supplement, encapsulation appears to be necessary to protecting RSV during digestion due to its degradation under pancreatic conditions (Tagliazucchi et al., 2010), as well as to control the dosage of RSV.

The present study focused on the effects of preparation parameters on the properties of niosomes containing RSV, properties of interest were the size distribution, chemical stability and entrapment efficiency. Mechanical agitation and sonication were employed as variable parameters in the preparation protocol.

2. Materials and methods

2.1. Materials

Trans-resveratrol (RSV), purity >99%, sorbitan monooleate (Span 80), sorbitan monostearate (Span 60) and cholesterol 95% were supplied by Sigma–Aldrich (Germany) and used without any previous treatment. Absolute ethanol (Baker analysed) was provided by Baker (The Netherlands). Deionized water was used in all experiments.

2.2. Preparation of niosomes

Niosomes containing RVS were prepared by agitation and sonication using non-ionic surfactants (Span 80 and Span 60).

A 416 mg/L stock solution of RSV in ethanol was prepared and stored at −20 °C. Working concentrations of RSV (50 mg/L) were obtained by dilution of the stock solution with deionized water, leading to a 12% (v/v) concentration of ethanol. These working solutions were stored at 4 °C protected from exposure to light.

Several surfactant concentrations in the range 1–4% (w/v) (Span 80 niosomes) and 0.5–2% (w/v) (Span 60-cholesterol niosomes) were investigated. Cholesterol was incorporated in the Span 60-niosomes formulation using a 1:1 surfactant/cholesterol weight ratio (6:7 M ratio). Surfactant/cholesterol molar ratios in the range of 6:4–7:3 are the best to obtain high entrapment efficiencies (Manosroi et al., 2003).

All batches of niosomes were prepared using a two-stage technique:

1. The solution of RSV in ethanol (6 mL) was mixed with (44 mL) of the dissolved surfactant either gently by magnetic stirring (500 rpm) for 1 h or vigorously with a homogenizer (SilentCrusher M., rotor model 22G, Heidolph, Germany), operating at 10,000 rpm for the Span 60-cholesterol niosomes, or at 15,000 rpm for Span 80 niosomes. Both homogenations proceeded for 15 min. Because of foam formation niosomes of Span 60-cholesterol could not be prepared at higher mechanical agitation speeds.
2. Subsequently mixing samples were sonicated for 30 min, in a sonicator (CY-500, Optic Ivymen System, Spain), using 50% amplitude, 500 W power and 20 kHz frequency.

2.3. Characterization of RSV niosomes

2.3.1. Determination of niosome size

Particle size ($d_{3,2}$) and volume size distribution analysis were carried out via dynamic light scattering (DSL) using a Malvern Mastersizer S Long Bench apparatus (Malvern Instruments, United Kingdom). Data points of niosomes size distributions are the average of at least three replicated data, with high reproducibility in the results (standard deviation usually less than 10%).

2.3.2. Morphology analysis of niosomes

Morphology analysis of niosomes was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM (Japan). A drop of the niosomal formulation was placed on a carbon-coated copper grid, and the sample excess was removed with filter paper. Then a drop of 2% (w/v) PTA (phosphotungsic acid solution) was applied to the carbon grid and left to stand for 2 min. Once the excess staining agent was removed with filter paper, the sample was air-dried and the thin film of stained niosomes was observed with the transmission electron microscope.

2.3.3. Stability of niosomes

The stability of niosomes was determined using light backscattering analysis in a Turbiscan Lab® Expert provided with an Ageing Station (Formulaction, France). The evaluated parameter was backscattering variation (ΔBS), which can be analyzed in each of three sections of the measuring cell: bottom, middle and top. Sample variations at the bottom and top of the measuring cell are linked to migration phenomena, and sedimentation and creaming respectively. Variations in the middle of the cell are due to changes in the size of niosomes.

Niosome samples in cylindrical glass tubes (40 mm measuring cells) were placed in the Turbiscan Ageing Station. Analyses of each sample were performed every 3 h for 15 days.

2.4. RSV entrapment efficiency of niosomes

The efficiency with which the niosomes entrapped RSV was determined by two methods.

- *Dialysis method:* A 3 mL sample of the niosomal suspension was placed in a dialysis tube (Novagen, D-Tube™ Dialyzer Maxi, MWCO 6–8 kDa, Millipore, USA), and the open end was plugged. The tube was then placed in a flask containing 100 mL of a 12% (v/v) solution of ethanol in water. The solution was gently mixed by magnetic stirring for 6 h. Then, the free RSV in dialysate was measured at 305 nm using a T80 UV/VIS Spectrometer (PG Instruments Ltd., United Kingdom). Selection of a dialysis membrane with no affinity for RSV is a key parameter to avoid errors in the determination of entrapment efficiency.
- *HPLC-RV method:* A niosome sample was centrifuged for 2 min at 12,000 rpm using a centrifuge Eppendorf 5415D (Germany). The supernatant was filtered using 0.45 μm PVDF syringe filters and analyzed by a HPLC (Series 1100, Hewlett Packard, USA). A reverse phase C_{18} column (Xbridge™ 5 μm particle size, 4.6 × 150 mm, Waters) was used and three different mobile phases were selected: water, acetonitrile and a mixture of methanol, acetonitrile, water and acetic acid (75:22.5:2:0.5, v/v). HPLC analyses were carried out at room temperature (20 °C) using a flow rate of 0.8 mL/min of the mobile phase.

2.5. In vitro release studies

The *in vitro* release of RSV from niosomes was determined using a dialysis membrane following Hu's method with minor variations (Hu and Rhodes, 1999). The dialysis membrane should have no affinity for RSV. Hence a Novagen, D-Tube™ Dialyzer Maxi, MWCO 6–8 kDa (Millipore, USA) was employed.

A closed dialysis tube containing 3 mL of niosomal formulation was placed in a flask containing 100 mL of a simulated gastric fluid (HCl solution 0.1 N, pH = 1.2 and 0.05 M NaCl). The flask was immersed in a thermostatic bath (Unitronic Orbital 320 OR, Selecta, Spain) and agitated at 37 °C with a swinging motion (40 U/min). In addition, 3 mL of hydroalcoholic solution with a 12% (v/v) of ethanol concentration and a known concentration of non-encapsulated RSV was placed into a dialysis tube under the same conditions to be used as a reference. Dialysate aliquots of 1 mL were taken at predetermined times. The RSV concentration was subsequently determined spectrophotometrically at 305 nm. Once the RSV analysis was performed, the sample was returned to the flask.

2.6. Statistical analysis

All data were expressed as the mean ± SD (standard deviation) of three independent experiments, and analysis of variance (ANOVA) was applied. Fisher's test ($p < 0.05$) was used to calculate the least significance difference (LSD) using a statistical software (Statgraphics v.15.2.06).

3. Results and discussion

Niosomal formulations with RSV were prepared with Span 60 and Span 80 as surfactants. Span 80 yields very stable niosomes with a narrow particle size distribution but they exhibit rather low entrapment efficiency (De Gier et al., 1968). By adding cholesterol to surfactant in a 1:1 weight ratio both stability and entrapment efficiency increased. In addition, surface of Span 80 and Span 60 niosomes is flexible due to their head group structure (Hayashi et al., 2011).

Samples of the RSV solution (6 mL) were added to the surfactant–cholesterol mixture in a 50 mL glass beaker. These samples were homogenized, and sonicated for 30 min using a sonicator equipped with a Ti probe. Sonication is a technique broadly used to obtain small unilamellar vesicles (SUVs) with a narrow size distribution. By contrast, use of other procedures such as mechanical agitation leads to production of multilamellar vesicles (MLVs) (Walde and Ichikawa, 2001). The physical properties, entrapment efficiency and *in vitro* RSV release were assessed for each of the resulting niosomal systems.

3.1. Optimization of sonication stage

Two sonication methods were considered: continuous mode and pulsed mode. The efficacy of using a pulsed sonication for sonication: paused times (in seconds) of 1:1, 2:2, 5:5 and 10:10 pulsed sonication were ascertained. The volumetric distribution of particle diameters of the niosomes formed with Span 80 (1% w/v), homogenized at 15,000 rpm for 15 min and sonicated for 30 min are shown in Fig. 1.

In all cases results reveal sonication decreases the niosome size, but continuous sonication mode gives a narrower size distribution than any of the pulsed mode. Consequently, continuous sonication will be used in subsequent experiments.

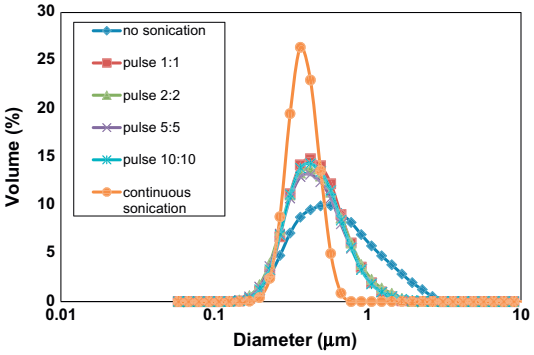

Fig. 1. Influence of sonication characteristics on the volume size distribution of RSV-niosomes.

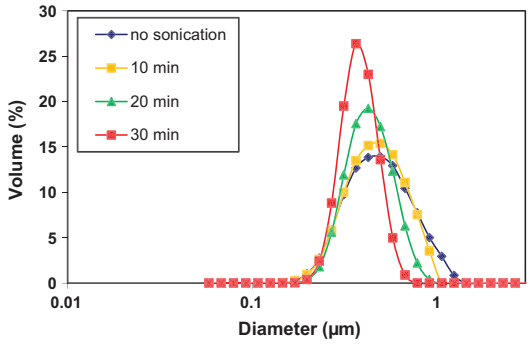

Fig. 2. Influence of continuous sonication time on the volume size distribution of RSV-niosomes. Sonication parameters: 500 W power, 50% amplitude and 20 kH frequency.

Table 1
Backscattering variation (ΔBS) for RSV-niosomes made of 1% (w/v) Span 80. Agitated at 15,000 rpm for 15 min (MLV) or agitated at 15,000 rpm for 15 min and sonicated continuously for 30 min (SUV).

Niosome	Bottom cell ΔBS_B	Middle cell ΔBS_M	Top cell ΔBS_T
MLV	−0.32 ± 0.07	−0.10 ± 0.01	3.78 ± 0.26
SUV	−0.45 ± 0.04	0.27 ± 0.02	0.31 ± 0.07

The influence of continuous sonication time on the niosome size is shown in Fig. 2 for the same preparation conditions: Span 80 (1% w/v) and previous homogenization at 15,000 rpm for 15 min.

Analysis of the results shown in Fig. 2 reveals that as the sonication time increases the average diameter decreases and the distribution of diameter is narrow. Hence, long sonication times lead to more stable niosomal systems.

The influence of sonication on niosomes stability was also determined by measurements of the backscattering variation, ΔBS, in the three zones of the Turbiscan cell: bottom (ΔBS_B), middle (ΔBS_M) and top (ΔBS_T). Results are shown in Table 1.

No substantial differences were observed in the ΔBS_B values for the two samples. The low values obtained for ΔBS_B also indicate that no sedimentation occurs in the bottom portion of the cell. Moreover, the low ΔBS_M values for the middle portion of the cell indicate that niosome size did not change significantly with time elapsed.

However, significant differences were found in ΔBS_T values. The ΔBS_T values increased with time for systems prepared without sonication. By contrast no significant increase was observed when sonication was employed. This result might be explained in terms of the smaller reduction in the sonicated niosomes. The reduction makes less the creaming effect.

Because longer sonication times may lead the equipment to overload, a sonication time of 30 min at 50% amplitude was selected as optimal value.

3.2. Niosomes characterization

The average size of the niosome, expressed as Sauter diameter ($d_{3,2}$), was measured before and after sonication. The polydispersity of niosome size distribution was expressed using the *span* parameter:

$$span = \frac{d_{90} - d_{10}}{d_{50}} \quad (1)$$

d_{90}, d_{50} and d_{10} are the diameters at which 90%, 50% and 10% of the volume distribution of niosomes are of smaller size, respectively. Lower span values are associated with narrower size distributions. $d_{3,2}$ and span values for all the systems prepared in this work are given in Table 2.

The influence of surfactant concentration on the $d_{3,2}$ values can be ascertained from inspection of the data shown in Fig. 3.

Analysis of the data in Table 2 and Fig. 3 indicates that SUV niosomes are always smaller than the corresponding MLV niosomes, that surfactant concentration influences only the size of the MLV niosomes, whereas there is not a direct relation between size of SUV niosomes and surfactant concentration. The niosomes made with Span 80 are smaller than those prepared with Span 60 and cholesterol, independent of the protocol employed. These results are consonant with previous works (Manconi et al., 2002) who reported that the vesicle size depends on the method of preparation, bilayer composition and biocomponent concentration.

Fig. 4 shows niosomes photomicrographs obtained by TEM microscopy.

As shown in Fig. 4, the SUV and MLV niosomes prepared with Span 80 reveal an ill-defined bilayer membrane, probably because of the double bond in the alkyl chain of the non-ionic Span 80 surfactant (De Gier et al., 1968). Its presence leads to high membrane permeability and a low density. Niosomes made with Span 60 and cholesterol exhibit a much better defined membrane characterized by a higher density, lower permeability and less leaky membrane (Betageri and Parsons, 1992; Rogerson et al., 1987) Consequently these niosomes have a higher entrapment efficiency and lower RSV release (see Section 3.5).

All these niosomes samples were prepared by vigorous agitation with a homogeneizer because the size distribution is narrower than using magnetic stirring, consequently the TEM images are more representative of the samples.

3.3. Stability of niosomes

The particle stability of the niosomes was studied by observing the backscattering variation (ΔBS). Changes in niosome size are linked to the ΔBS_M measured in the middle zone of the Turbiscan cell. An increase of niosome diameter indicates that coagulation/flocculation has taken place, leading to destabilization of the sample. ΔBS_M values are shown in Table 3.

ΔBS_M values are low for systems containing SUV niosomes made with Span 80 either agitated at 15,000 or at 500 rpm as the first preparation stage. No size variation was detected during storage at temperatures of 30 °C for 15 days. Niosomes made with Span 60-cholesterol show larger ΔBS_M values and hence lower stability. The negative ΔBS_M values reveal a backscattering decrease, resulting from an increase in the size of niosomes. The variation of average size for those niosomes agitated at 500 rpm before sonication was calculated using commercial software (Formulaction, France). The result was an increase in the diameter of 0.01 μm/day. However, for the niosomes prepared using other protocols the increase in niosome size was negligible.

Figs. 5 and 6 show two different types of ΔBS behavior. Fig. 5 corresponds to one of the most stable systems: SUV niosomes prepared using 2% (w/v) of Span 80. Fig. 6 corresponds to one of the less stable systems: SUV niosomes prepared using 2% (w/v) of Span 60 and cholesterol in a weight ratio of 1:1.

As Fig. 5 indicates there is very little variation in backscattering. By contrast, inspection of Fig. 6 reveals a marked increase in ΔBS_B

Table 2
Average diameter ($d_{3,2}$) and span values of the size distribution in MLV and SUV RSV-niosomes. 1 h agitation time at 500 rpm or 15 min agitation time at 10,000 or 15,000 rpm.

Component	Concentration	Agitation (rpm)	$d_{3,2}$ (μm)		Span	
			MLV	SUV	MLV	SUV
Span 80	1% (w/v)	500	5.11 ± 0.49	0.40 ± 0.02	2.27 ± 0.17	1.18 ± 0.07
Span 80	1% (w/v)	15,000	0.39 ± 0.02	0.34 ± 0.01	1.11 ± 0.07	0.58 ± 0.03
Span 80	2% (w/v)	500	5.84 ± 0.51	0.41 ± 0.01	2.31 ± 0.19	2.09 ± 0.08
Span 80	2% (w/v)	15,000	0.40 ± 0.02	0.37 ± 0.01	1.64 ± 0.09	1.01 ± 0.03
Span 80	3% (w/v)	500	8.47 ± 0.71	0.43 ± 0.02	2.14 ± 0.19	1.48 ± 0.04
Span 80	3% (w/v)	15,000	0.39 ± 0.01	0.38 ± 0.01	0.98 ± 0.08	0.98 ± 0.04
Span 80	4% (w/v)	500	11.88 ± 0.91	0.44 ± 0.02	1.88 ± 0.16	1.72 ± 0.08
Span 80	4% (w/v)	15,000	0.38 ± 0.02	0.36 ± 0.02	1.56 ± 0.14	0.70 ± 0.06
Span 60	0.5% (w/v)	500	1.86 ± 0.12	0.91 ± 0.02	4.52 ± 0.31	4.39 ± 0.28
Cholesterol	0.5% (w/v)					
Span 60	0.5% (w/v)	10,000	0.92 ± 0.05	0.86 ± 0.02	3.02 ± 0.24	2.54 ± 0.12
Cholesterol	0.5% (w/v)					
Span 60	1% (w/v)	500	4.44 ± 0.37	0.86 ± 0.02	4.06 ± 0.33	4.83 ± 0.39
Cholesterol	1% (w/v)					
Span 60	1% (w/v)	10,000	1.05 ± 0.09	0.81 ± 0.03	3.98 ± 0.25	6.78 ± 0.46
Cholesterol	1% (w/v)					
Span 60	1.5% (w/v)	500	4.55 ± 0.35	0.89 ± 0.03	5.21 ± 0.41	2.75 ± 0.19
Cholesterol	1.5% (w/v)					
Span 60	1.5% (w/v)	10,000	1.52 ± 0.07	0.88 ± 0.02	13.19 ± 0.92	11.83 ± 1.03
Cholesterol	1.5% (w/v)					
Span 60	2% (w/v)	500	4.48 ± 0.41	0.84 ± 0.02	5.65 ± 0.36	2.46 ± 0.18
Cholesterol	2% (w/v)					
Span 60	2% (w/v)	10,000	1.54 ± 0.11	0.82 ± 0.04	11.16 ± 0.93	10.03 ± 0.93
Cholesterol	2% (w/v)					

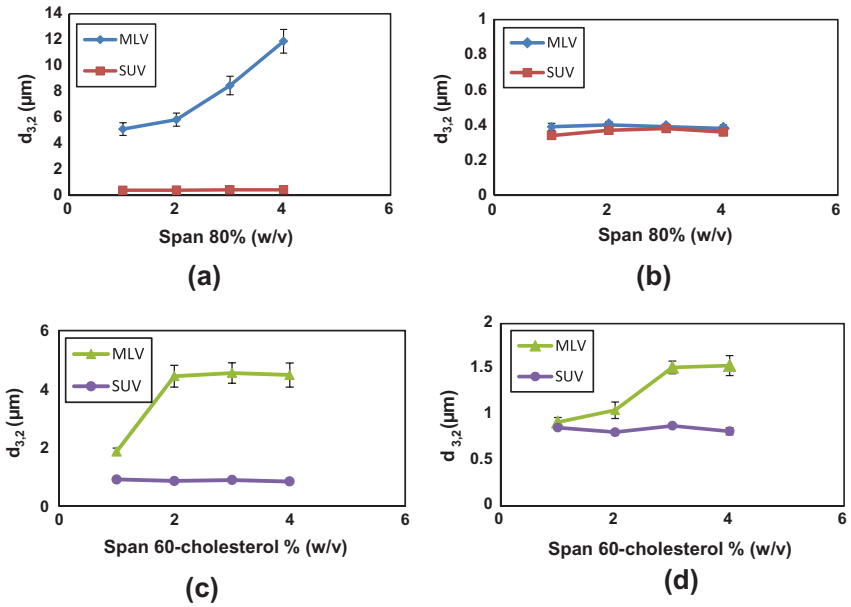

Fig. 3. Influence of surfactant concentration on the average diameter ($d_{3,2}$) of RSV-niosomes (a) stirring at 500 rpm for 1 h (MLV), agitated at 500 rpm for 1 h followed by 30 min of continuous sonication (SUV); (b) stirring at 15,000 rpm for 15 min (MLV), stirring at 15,000 rpm for 15 min followed by 30 min of continuous sonication (SUV); (c) stirring at 500 rpm for 1 h (MLV), stirring at 500 rpm for 1 h followed by 30 min of continuous sonication (SUV); (d) stirring at 10,000 rpm for 15 min (MLV), stirring at 10,000 rpm for 15 min followed by 30 min of continuous sonication (SUV).

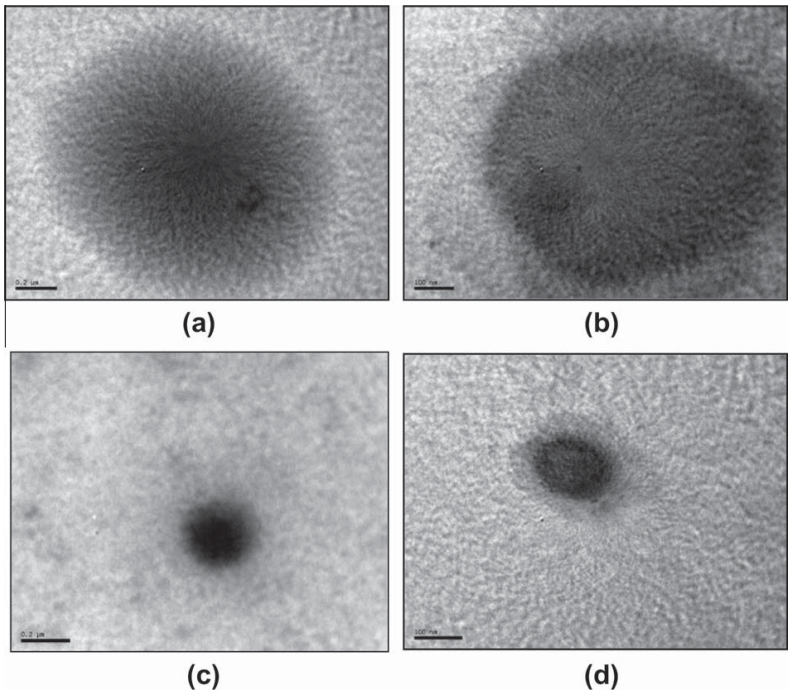

Fig. 4. Photomicrographs of RSV-niosomes for several preparation conditions: (a) 1% (w/v) Span 80 agitated at 15,000 rpm for 15 min (MLV); (b) 1% (w/v) Span 60-cholesterol (1:1 w) agitated at 10,000 rpm for 15 min (MLV); (c) 1% (w/v) Span 80 agitated at 15,000 rpm for 15 min followed by 30 min of continuous sonication (SUV); (d) 1% (w/v) Span 60-cholesterol (1:1 w) agitated at 10,000 rpm for 15 min followed by 30 min of continuous sonication (SUV). Bars: 0.2 µm (a and c) and 100 nm (b and d).

Table 3
Backscattering variation in the middle of the measuring cell for SUV RSV-niosomes. 1 h of agitation at 500 rpm or 15 min of agitation at 15,000 rpm.

Component	Concentration	Agitation (rpm)	ΔBS_M (%)
Span 80	1% (w/v)	500	0.28 ± 0.02
Span 80	1% (w/v)	15,000	0.27 ± 0.02
Span 80	2% (w/v)	500	0.37 ± 0.02
Span 80	2% (w/v)	15,000	0.18 ± 0.02
Span 80	3% (w/v)	500	0.17 ± 0.01
Span 80	3% (w/v)	15,000	0.31 ± 0.02
Span 80	4% (w/v)	500	0.72 ± 0.04
Span 80	4% (w/v)	15,000	0.39 ± 0.02
Span 60	0.5% (w/v)	500	−8.35 ± 0.56
Cholesterol	0.5% (w/v)		
Span 60	0.5% (w/v)	10,000	−4.51 ± 0.41
Cholesterol	0.5% (w/v)		
Span 60	1% (w/v)	500	−6.27 ± 0.51
Cholesterol	1% (w/v)		
Span 60	1% (w/v)	10,000	−2.33 ± 0.33
Cholesterol	1% (w/v)		
Span 60	1.5% (w/v)	500	−5.94 ± 0.49
Cholesterol	1.5% (w/v)		
Span 60	1.5% (w/v)	10,000	−2.81 ± 0.23
Cholesterol	1.5% (w/v)		
Span 60	2% (w/v)	500	−7.42 ± 0.52
Cholesterol	2% (w/v)		
Span 60	2% (w/v)	10,000	−1.20 ± 0.11
Cholesterol	2% (w/v)		

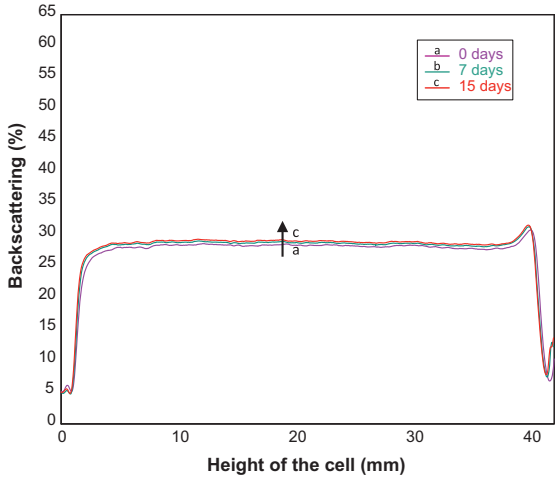

Fig. 5. Backscattering profiles for SUV RSV-niosomes made of Span 80 (2% w/v).

due to sedimentation phenomena, and a decrease in ΔBS_M, which results in an increase in the average size of niosome. Consequently, the Span 80 niosomes are more stable than the Span 60-cholesterol niosomes. Moreover, high speed agitation prior to sonication enhances the stability of niosomal systems.

3.4. RSV entrapment efficiency

The niosomes containing RSV were separated from untrapped RSV using centrifugation at 12,000 rpm for 2 min using an Eppendorf 5415D centrifuge (Germany). The amount of untrapped RSV was determined by RP-HPLC and the dialysis method was used to confirm these results. The values of entrapment efficiency are shown in Table 4.

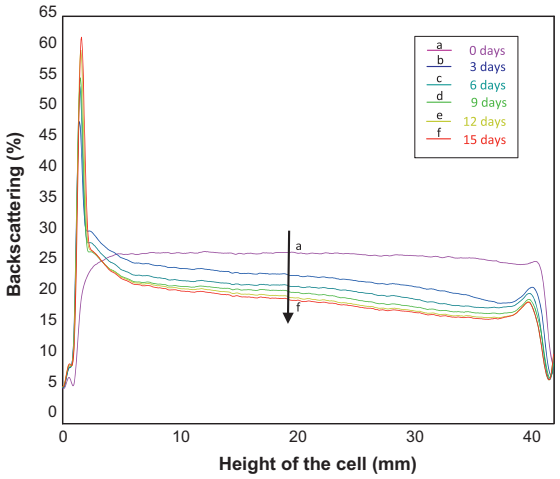

Fig. 6. Backscattering profiles for SUV RSV-niosomes made of Span 60 (2% w/v) and cholesterol in a weight ratio of 1:1.

Table 4
RSV entrapment efficiency of SUV niosomes. 1 h of agitation at 500 rpm or 15 min of agitation at 15,000 rpm. Sonication time: 30 min.

Component	Concentration	Agitation (rpm)	Entrapment efficiency (%)
Span 80	1% (w/v)	500	12.41 ± 0.41
Span 80	1% (w/v)	15,000	~0
Span 80	2% (w/v)	500	12.22 ± 0.76
Span 80	2% (w/v)	15,000	~0
Span 80	3% (w/v)	500	13.66 ± 0.52
Span 80	3% (w/v)	15,000	~0
Span 80	4% (w/v)	500	11.88 ± 0.44
Span 80	4% (w/v)	15,000	~0
Span 60	0.5% (w/v)	500	32.48 ± 1.78
Cholesterol	0.5% (w/v)		
Span 60	0.5% (w/v)	10,000	2.77 ± 0.19
Cholesterol	0.5% (w/v)		
Span 60	1% (w/v)	500	35.86 ± 3.43
Cholesterol	1% (w/v)		
Span 60	1% (w/v)	10,000	8.33 ± 1.27
Cholesterol	1% (w/v)		
Span 60	1.5% (w/v)	500	40.96 ± 4.34
Cholesterol	1.5% (w/v)		
Span 60	1.5% (w/v)	10,000	16.82 ± 2.19
Cholesterol	1.5% (w/v)		
Span 60	2% (w/v)	500	43.74 ± 0.93
Cholesterol	2% (w/v)		
Span 60	2% (w/v)	10,000	14.50 ± 1.95
Cholesterol	2% (w/v)		

For the Span 80 niosomes prepared using agitation at 15,000 rpm before sonication the entrapment efficiency of RSV is practically zero. When this system was magnetically agitated at 500 rpm, the niosomes were able to encapsulate a small amount of RSV, around 12% (w/w).

The Span 60-cholesterol niosomes have greater entrapment efficiency of RSV. The efficiency increases when low mechanical agitation speeds are used in the first stage of the preparation protocol. In Fig. 7 the entrapment efficiency is plotted as a function of the surfactant concentration used in niosomes formulation.

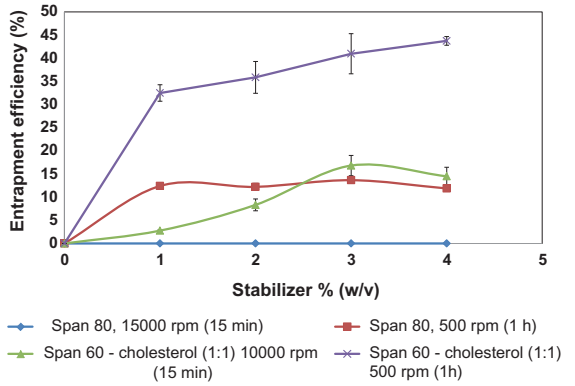

Fig. 7. Influence of surfactant concentration (Span 80 niosomes) and surfactant + cholesterol concentration in a 1:1 weight ratio (Span 60 – cholesterol niosomes) in the efficiency for entrapment of RSV in SUV niosomes (30 min sonication).

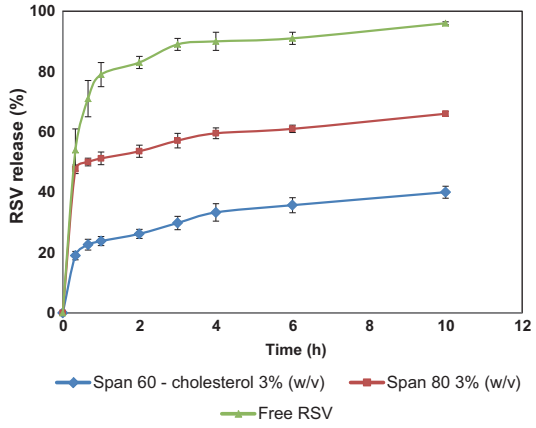

Fig. 8. RSV release from SUV niosomes made of Span 60 – cholesterol (1:1 w/w) or Span 80, and free RSV.

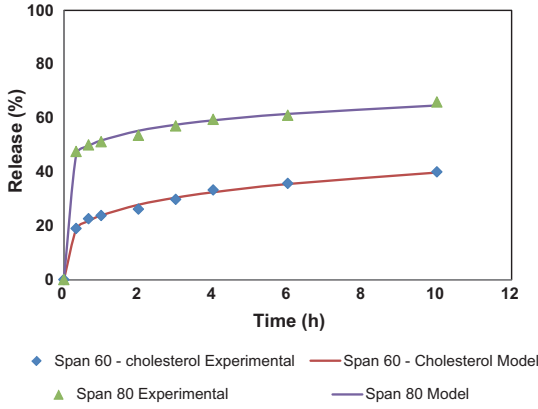

Fig. 9. Fit of RSV release data from SUV niosomes to Korsmeyer–Peppas model.

Table 5
K and *n* parameters of the Korsmeyer–Peppas model for RSV release from SUV niosomes.

	K	*n*	r^2
Span 60 – cholesterol (1:1 w/w) 3% (w/v)	23.9	0.222	0.996
Span 80 3% (w/v)	51.7	0.098	0.998

The RSV entrapment efficiency is higher for the Span 60-cholesterol niosomes when they are prepared using gentle agitation (500 rpm) followed by sonication. Moreover, the RSV encapsulation capacity increases at higher concentrations of surfactant-cholesterol.

3.5. RSV release from niosomes

The release of RSV from niosomal formulations was determined by *in vitro* studies in which the formulations were contacted for 10 h at 37 °C with a simulated gastric fluid (HCl solution 0.1 N, pH = 1.2 and 0.05 M NaCl). A 3% (w/v) concentration was selected for both the Span 80 niosomes and Span 60-cholesterol niosomes. These concentrations were selected because of their high RSV entrapment efficiencies. Samples were agitated at 500 rpm for 1 h and sonicated for 30 min using 50% amplitude. The RSV release was determined spectrophotometrically at 305 nm. Results are shown in Fig. 8.

RSV release after 10 h was 66% (w/w) and 40% (w/w) for niosomes made of Span 80 and Span 60-cholesterol, respectively. The difference might be attributed to the presence of a double bond in the alkyl chain of the Span 80 non-ionic surfactant (De Gier et al., 1968) and the membrane stabilization and low permeability produced by cholesterol (Betageri and Parsons, 1992).

A kinetic analysis of RSV release was carried out using the Korsmeyer–Peppas model. This model is particularly suitable when the release mechanism is not well established or if several release phenomena are involved (Hayashi et al., 2005).

$$F = Kt^n \quad (2)$$

K is a parameter that depends on the structural and geometric characteristics of the RSV-containing niosomes, *n* is the release order, (an indication of the release mechanism), and *F* represents the fractional release of RSV at time *t*. Fig. 9 contains RSV release data plotted according to the Korsmeyer–Peppas model and the parameters *K* and *n* that fit best the model are indicated in Table 5.

If *n* lies in the range between 0 and 0.5, the system follows a Fickian diffusion transport mechanism, while if *n* lies between 0.5 and 1 convective mass transfer is the pertinent mechanism (anomalous transport) (Yedurkar et al., 2007; Costa and Sousa-Lobo, 2001).

Release of RSV from Span 60-cholesterol and Span 80 niosomes follows a Fickian diffusion model.

4. Conclusions

Niosomes prepared from Span 80 and Span 60-cholesterol are able to encapsulate RSV. Average size, stability and RSV entrapment efficiency of niosomes are linked not only to their composition but also to the protocol employed in preparing the niosomes.

High speed mechanical agitation produces niosomes with a smaller average size and a narrower size distribution. However the resulting entrapment efficiency is less than that for niosomes prepared using agitation at low speed. The use of sonication as a second stage decreases the average size of the niosomes.

Higher RSV entrapment efficiency was observed for SUV niosomes made using Span 60-cholesterol in a 1:1 weight ratio and

a concentration of 4% (w/v). The amount of encapsulated RSV is 43.7%, showing a slower release than Span 80-niosomes.

Proper selection of niosome formulation and preparation method are required depending on the characteristics of final product, being the most important parameters the entrapment efficiency and RSV release.

Acknowledgements

This work was supported by the MICINN, Spain, under the Grant MICINN-10-CTQ2010-20009-C02-01. D. Pando acknowledges receipt of a graduate fellowship from Severo Ochoa Program (Principado de Asturias, Spain). The authors thank Prof. Charles G. Hill (University of Wisconsin-Madison) for fruitful discussions.

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II. Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol

Several papers have been published in the last decades with the main objective to enhance dermal and transdermal drug delivery. Vesicles are one of the most studied nanocarrier systems because they can increase the drug residence time in dermis and epidermis, reducing its systemic absorption and significantly enhancing the drug delivery into or through skin.

In this work resveratrol (RSV) was incorporated into niosomes and liposomes, in order to protect it from degradation and to enhance its skin delivery. The ex-vivo RSV percutaneous absorption was performed on newborn pig skin using vertical Franz diffusion cells.

Cholesterol was added as membrane stabilizer and the influence of vesicle composition on their physicochemical properties and stability was evaluated. Phosphatidylcholine was used for liposomes formulation while niosomes were formulated with two suitable skin-compatible oleins used in pharmaceutical formulations as surfactants.

Article 2. D. Pando, C. Caddeo, M. Manconi, A.M. Fadda, C. Pazos. *Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol*.

Journal of Pharmacy and Pharmacology 65 (2013) 1158-1167 DOI:10.1111/jphp.12093

Personal contribution to work

I carried out this work during my stay at the *Dipartamento di Scienze della Vita e dell'Ambiente, Sezione di Scienze del Farmaco*, Università di Cagliari (Italia). After a training period I conducted the preparation and characterization of vesicles, and the *ex vivo* studies on pig skin in Franz diffusion cells. Results are reported in the attached paper, which was carefully written in collaboration with Drs. Carla Caddeo, Maria Manconi and Anna Maria Fadda, and under the supervision of Prof. Carmen Pazos.

II. Nanodiseño de vesículas de oleína para la liberación tópica del antioxidante resveratrol

En las últimas décadas se han publicado diversos trabajos cuyo principal objetivo se dirigía a mejorar la liberación de fármacos por vía dérmica y transdérmica. Las vesículas son uno de los sistemas de liberación más estudiados debido a que pueden aumentar el tiempo de residencia del principio activo en la dermis y en la epidermis, reduciendo su absorción sistémica y mejorando significativamente la liberación del mismo sobre o a través de la piel.

En este trabajo, se realizó la encapsulación del resveratrol (RSV) en niosomas y liposomas, con el fin de protegerlo de la degradación y mejorar su liberación en la piel. La absorción percutánea *ex vivo* del RSV se estudió en piel de cerdo recién nacido usando celdas verticales de difusión Franz.

Como estabilizante de membrana se utilizó colesterol y se estudió la influencia de la composición de la vesícula en sus propiedades fisicoquímicas y estabilidad. La formación de liposomas se realizó con fosfatidilcolina, mientras que los niosomas se formularon con dos oleínas compatibles con la piel, usadas como tensioactivos en productos farmacéuticos.

Artículo 2. D. Pando, C. Caddeo, M. Manconi, A.M. Fadda, C. Pazos. *Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol*

Journal of Pharmacy and Pharmacology 65 (2013) 1158-1167. DOI:10.1111/jphp.12093

Contribución personal al trabajo

Este trabajo lo realicé durante mi estancia en el *Dipartamento di Scienze della Vita e dell'Ambiente, Sezione di Scienze del Farmaco*, Università di Cagliari (Italia). Después de un periodo inicial de aprendizaje de las técnicas experimentales, me encargué de la preparación de las vesículas, de su caracterización y de los estudios *ex vivo* con piel de cerdo en celdas Franz. Los resultados se reflejan en el artículo adjunto, cuyo manuscrito inicial elaboré con la colaboración de las Dras. Carla Caddeo Maria Manconi y Anna Maria Fadda, así como la supervisión de mi directora de Tesis, Prof. Carmen Pazos.

JPP JOURNAL OF Pharmacy and Pharmacology
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JPP Journal of Pharmacy And Pharmacology
Research Paper

Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol

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Keywords
dermal application; liposome; niosome; resveratrol

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Received January 9, 2013
Accepted May 9, 2013

doi: 10.1111/jphp.12093

Abstract

Objectives The ex-vivo percutaneous absorption of the natural antioxidant resveratrol in liposomes and niosomes was investigated. The influence of vesicle composition on their physicochemical properties and stability was evaluated. Liposomes containing resveratrol were formulated using soy phosphatidylcholine (Phospholipon90G). Innovative niosomes were formulated using mono- or diglycerides: glycerol monooleate (Peceol) and polyglyceryl-3 dioleate (Plurol OleiqueCC), respectively, two suitable skin-compatible oleins used in pharmaceutical formulations as penetration enhancers.

Methods Small, negatively charged vesicles with a mean size of approximately 200 nm were prepared. The accelerated stability of vesicles was evaluated using Turbiscan Lab Expert, and the bilayer deformability was also assessed. Ex-vivo transdermal experiments were carried out in Franz diffusion cells, on newborn pig skin, to study the influence of the different vesicle formulations on resveratrol skin delivery.

Key findings Results indicated a high cutaneous accumulation and a low transdermal delivery of resveratrol, especially when Peceol niosomes were used.

Conclusions Overall, niosomes formulated with Plurol oleique or Peceol showed a better behaviour than liposomes in the cutaneous delivery of resveratrol.

Introduction

Several innovative technological approaches have been made in recent decades to enhance dermal and transdermal drug delivery. Liposomes and niosomes are the most studied nanocarrier systems for their ability to enhance drug delivery into or through skin, as well as to deliver the drug to its therapeutic target, while reducing drug toxicity.[1–4] Liposomes and niosomes can increase the residence time of the drug in the stratum corneum and epidermis, reducing the systemic absorption of the drug.[5] Furthermore, these vesicles improve the horny layer properties both by reducing transepidermal water loss and by replacing lost skin lipids, improving the smoothness of the skin.[6] Niosomes have two main advantages over liposomes: higher chemical stability and lower cost.[7] Moreover, surfactants in niosomes contribute to the overall penetration enhancement of compounds primarily by adsorption at interfaces, by interacting with biological membranes and by altering the barrier function of the stratum corneum, as a result of reversible lipid modification.[8] Hence, niosomes are considered a good alternative to liposomes and nowadays they are playing an important role in drug delivery.

Resveratrol is a natural polyphenol mainly found in grapes, nuts and berries. Resveratrol shows geometric isomerism, but only trans-resveratrol (trans-3,4,5-trihidroxystilbene) offers several biological effects, such as anticancer activity due to its anti-ageing effect, antioxidant activity, anti-inflammatory activity and neuroprotective effect.[9–16] Since resveratrol has strong antioxidant properties, and oxidative stress is believed to be a critical factor in a variety of cutaneous conditions including skin cancers, it is a very interesting drug to be incorporated in dermal products.[17,18]

The aim of this work was to incorporate resveratrol into liposomes and niosomes to improve its delivery to the skin,

besides protecting it from photodegradation. To this purpose, liposomes were prepared using soy phosphatidylcholine (Phospholipon90G, P90), while innovative niosomes were made using mono- or diglycerides: Peceol (PEC) and Plurol oleique CC (PLU), two interesting and useful surfactants. They are, respectively, a mixture of glycerol monooleate and other fatty acids (monoolein), and polyglyceryl-3 dioleate and other fatty acids (diolein). They are biodegradable, nontoxic, biocompatible excipients, commonly used in pharmaceutical formulations as penetration enhancers for their ability to temporarily and reversibly disrupt the ordered lamellar structure of the stratum corneum.[19] Cholesterol was added as a stabilizer of the vesicular membrane and its amount was optimized experimentally. The ex-vivo transdermal delivery and cutaneous targeting of resveratrol-loaded vesicles were evaluated using vertical Franz diffusion cells and newborn pig skin.

Materials and Methods

Materials

Resveratrol purity > 99%, was purchased from Sigma-Aldrich (Milan, Italy). Glycerol monooleate (PEC) and polyglyceryl-3 dioleate (PLU) were a gift from Gattefossè (Saint Priest, France). Cholesterol was purchased from Sigma-Aldrich (Milan, Italy) and enriched soy phosphatidylcholine (P90) was a gift from AVG (Milan, Italy) and Lipoid GmbH (Ludwigshafen, Germany).

Vesicle preparation

Resveratrol (1 mg/ml) liposome and niosome composition is reported in Table 1. All components were weighed in a glass flask, one after the other, and 5 ml phosphate buffer saline (PBS, pH = 7) was added.[20,21] The suspensions were sonicated (10 cycles of 5 s intercalated with pause of 2 s; 13 μm of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK) and clear opalescent dispersions were obtained.

Vesicle characterization

Morphological analysis of vesicles was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM. A drop of the vesicular formulation was placed on a carbon-coated copper grid, and the sample excess was removed using a piece of filter paper. A drop of phosphotungstic acid solution (2% w/v) was then applied to the carbon grid and left for 2 min. Once the excess of staining agent was removed by absorbing with the filter paper, the sample was air-dried and the thin film of stained niosomes observed under the TEM.

Vesicles were also characterized by measuring the Brownian motion of the particles in samples using dynamic light scattering for mean size and polydispersity index (PI: a measure of the width of the size distribution) with the Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). Zeta potential was estimated using the Zetasizer Nano ZS by means of the M3-PALS (phase analysis light scattering) technique, which measures the particle electrophoretic mobility. High absolute values of zeta potential indicate electrical repulsion between vesicles, which is linked to high stability. Before the mean size and zeta potential measurements, samples (10 μl) were diluted in PBS (5 ml).

Stability of vesicles was determined by light scattering analysis in the Turbiscan Lab Expert provided with an Ageing Station (Formulaction, France). Measurements were performed using a pulsed near infrared LED set at a

Table 1 Niosomes and liposomes composition (mg/ml)

Sample name	P90 (mg/ml)	Plurol Oleique CC (mg/ml)	Peceol (mg/ml)	Cholesterol (mg/ml)
P90-liposomes	30	–	–	–
PLU-niosomes 30 : 10	–	30	–	10
PLU-niosomes 30 : 20	–	30	–	20
PLU-niosomes 30 : 30	–	30	–	30
PLU-niosomes 40 : 10	–	40	–	10
PLU-niosomes 40 : 20	–	40	–	20
PLU-niosomes 40 : 30	–	40	–	30
PLU-niosomes 40 : 40	–	40	–	40
PEC-niosomes 30 : 10	–	-	30	10
PEC-niosomes 30 : 20	–	-	30	20
PEC-niosomes 30 : 30	–	-	30	30
PEC-niosomes 40 : 10	–	-	40	10
PEC-niosomes 40 : 20	–	-	40	20
PEC-niosomes 40 : 30	–	-	40	30
PEC-niosomes 40 : 40	–	-	40	40

P90, Phospholipon90G (enriched soy phosphatidylcholine); PLU, Plurol Oleique CC (polyglyceryl-3 dioleate); PEC, Peceol (glycerol monooleate).

wavelength of 880 nm. The light transmitted (T) and backscattered (BS) was recorded. Backscattering variation Δ(BS) was the evaluated parameter, because T was nil for the samples. Δ(BS) can be analysed in three parts of the measuring cell: bottom, middle and top. Vesicular suspensions were put into cylindrical glass tubes (40 mm measuring cells) and placed in the Ageing Station. The analysis of each sample was carried out every 3 h for 15 days.

Entrapment efficiency

Loaded resveratrol was separated from the unloaded drug by dialysis. A 2-ml sample was put into a dialysis bag, which was immersed in 1000 ml PBS at room temperature and stirred at 500 rev/min for 2 h. Dialysed and nondialysed samples were diluted 1 : 1000 (v/v) with methanol to allow rupture of the vesicle membrane and to extract resveratrol from the vesicles. Resveratrol content was assayed at 306 nm using a chromatograph Alliance 2690 (Waters, Milan, Italy). The analytical column was an XBridge C18, 5 μm, 4.6 × 150 mm (Waters, Milan, Italy). The mobile phase was a mixture of methanol, acetonitrile, water and acetic acid (75 : 22.5 : 2.4 : 0.1, v/v) at a flow rate of 0.8 ml/min.

Deformation index determination

Comparative measurements of deformability of niosome and liposome bilayers were carried out by extrusion. A Liposofast (Avestin, Canada) extruder equipped with a 0.05-μm pore size polycarbonate track-etched membrane (Nuclepore, Whatman) was used. The vesicle dispersion was extruded at constant pressure (4 bar) through the membrane. Vesicle deformability was expressed in terms of deformation index (DI) according to equation 1.[22, 23]

$$\mathrm{DI} = J\left(\frac{d_0}{p}\right)\left(\frac{d_0}{|d_0 - d_1|}\right) \quad (1)$$

where J is the amount of suspension recovered after extrusion, d_0 and d_1 are the mean diameter of vesicles before and after extrusion, respectively, and p is the pore size of the membrane.

Ex-vivo skin penetration and permeation studies

Experiments were performed nonocclusively using vertical Franz cells with an effective diffusion area of 0.785 cm^2. Experiments were carried out using the skin of newborn Goland-Pietrain hybrid pigs (~1.2 kg) that had died of natural causes, provided by a local slaughterhouse.. The skin, previously frozen at-80°C, was pre-equilibrated in saline at 25°C for 1 h before the experiments, then sandwiched securely between donor and receptor compartments of the Franz cells with the stratum corneum side facing the donor compartment. The receptor container, thermostated at 37 ± 1°C, was filled with 5.5 ml saline (0.9% w/v NaCl), which was continuously agitated with magnetic stirring. A 100-μl sample was applied onto the skin, in the donor compartment ($n = 6$ per formulation). At regular intervals the receiver solution was withdrawn and replaced by fresh saline. After 8 h, the skin was removed from the Franz cells and gently washed. To determine the amount of resveratrol accumulated on each layer of the skin (stratum corneum, dermis and epidermis) a separation of these layers was carried out. Stratum corneum was removed by stripping with adhesive tape (Tesa AG, Hamburg, Germany). The method had been validated previously by histological examination of stripped skin.[20] Epidermis was separated from dermis with a surgical scalpel. Each skin layer, tape strips, epidermis and dermis, were separately placed in a flask with methanol to extract resveratrol, helped with sonication for 4 min. Receiver compartment samples were lyophilized and then methanol was added to extract resveratrol. Drug content in skin layers and receiver compartment samples was quantified by HPLC.

Statistical analysis of data

Results are expressed as the mean ± standard deviation. Parametric and nonparametric analysis of variance (analysis of variance and Kruskal–Wallis, respectively) and Bartlet's test for homogeneity of variance were performed using SPSS version 17.0 for Windows (SPSS Inc., USA). Post-hoc testing ($P < 0.05$) for multiple comparisons ($n = 6$) was performed by Scheffe and Dunnett's T3 tests.

Results and Discussion

Vesicle characterization

In this work, liposomes were made from soy phosphatidylcholine, and niosomes were prepared using two glycerides PEC, a monoolein, and PLU, a diolein. Thus, niosomes combine the ability of oleins to temporarily alter the structure of the stratum corneum with the ability of vesicles to carry the drug across the skin.[16] Two different amounts of oleins were combined with cholesterol (olein : cholesterol weight ratios) to obtain stable and small vesicles. The resveratrol-loaded niosomes with the best physicochemical properties were selected to perform (trans)dermal experiments in comparison with conventional liposomes.

Vesicle formation and morphology were confirmed by TEM for all the prepared samples. Figure 1 shows four negative stain micrographs of resveratrol-loaded niosomes. The actual formation of all niosomes was confirmed. Micrographs showed circular and dark structures corresponding to spherical niosomes of approximately 200 nm.

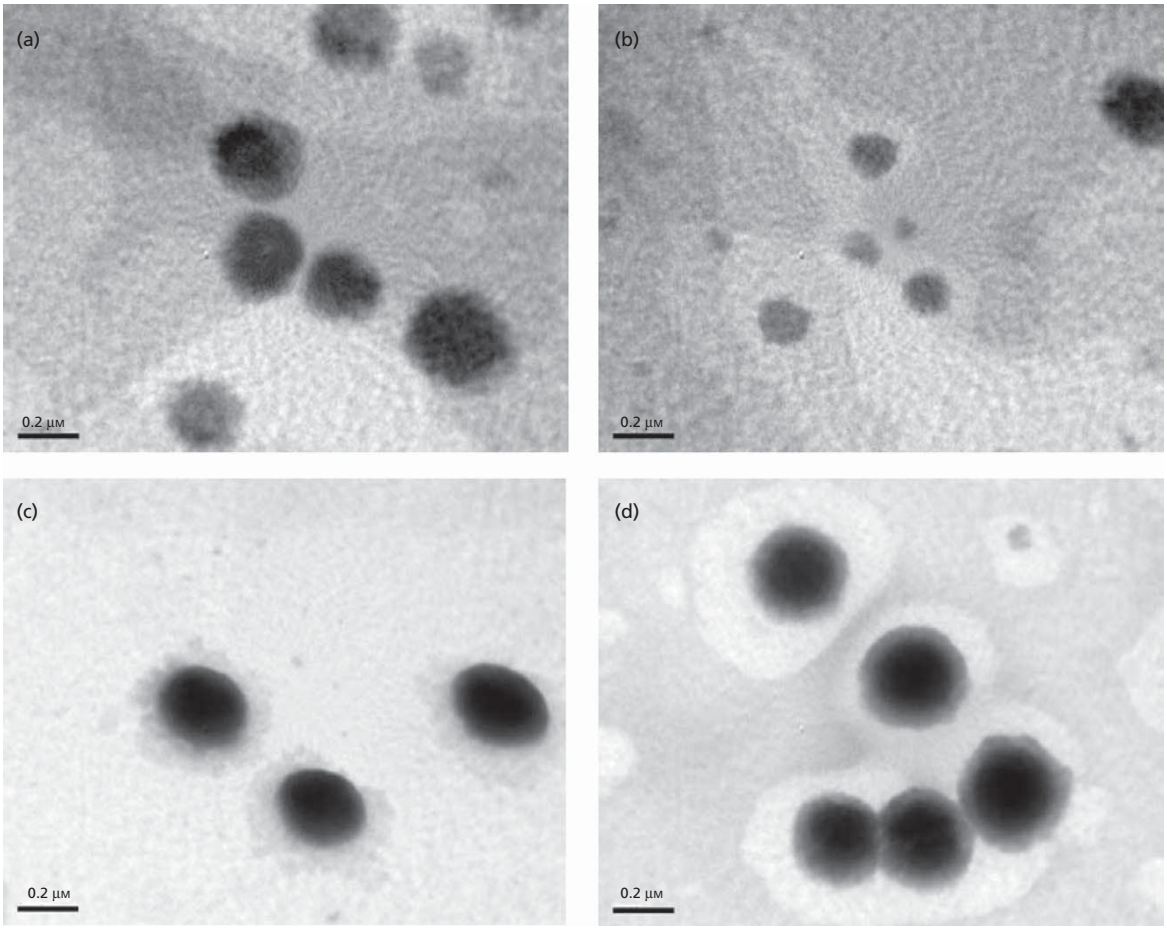

Figure 1 Niosome micrographs. (a) Plurol Oleique CC (polyglyceryl-3 dioleate; PLU)-niosomes 30 : 20, (b) PLU-niosomes 30 : 30, (c) Peceol (glycerol monooleate; PEC)-niosomes 30 : 20 and (d) PEC-niosomes 30 : 30.

Dark-stained niosomes were obtained due to the strong interactions between the surfactants and the phosphotungstic acid, which was able to selectively deposit electrons in the sample and enhance structural details. Oleins and phosphotungstic acid covered the vesicles structure making impossible the evaluation of their lamellarity.

All vesicle formulations were characterized to determine mean size, size distribution, zeta potential, and backscattering variation (Table 2).

Liposomes are nano-sized lamellar vesicles composed of phospholipid bilayers, while niosomes are formed by nonionic surfactants. Among lamellar vesicles, liposomes have been extensively investigated in the pharmaceutical field as safe and effective drug carriers for skin delivery. In particular, a high dermal efficacy has been proved for several liposomal formulations loading different drugs. Taking into account their similarity with niosomes and their large diffusion as skin delivery systems, liposomes were used as the control in this study. Control liposomes showed an average size of approximately 187 nm, a negative zeta potential (~−18 mV) and a large size distribution (PI > 0.3). PLU- and PEC-niosomes could be obtained only in the presence of cholesterol (10–40 mg/ml), using different amounts of surfactants (30 or 40 mg/ml). There were no important differences in average diameter between PLU-niosomes and P90-liposomes ($P > 0.05$), except for PLU-niosomes 30 : 30 that showed a lower mean diameter ($P < 0.05$). Control liposomes showed a smaller size than that of PEC-niosomes ($P < 0.05$). Zeta potential values were less negative in liposomes than in niosomes (~−40 mV, $P < 0.01$), thus conferring higher stability against aggregation to niosomes. The olein structure affected the vesicle average diameter, PLU being a diolein, with two apolar chains and a large hydrophilic head (hydrophilic–lipophilic balance (HLB) =

Table 2 Average size, polydispersity index, zeta potential and backscattering variation of liposomes and PLU- and PEC-niosomes containing different amounts of cholesterol

Samples	Size (nm)	Polydispersity index	Zeta potential (mV)	$\Delta(BS)_M$ (%)
P90-liposomes	187 ± 5	0.35	–18 ± 3	+0.8
PLU-niosomes 30 : 10	178 ± 6	0.28	–45 ± 2	–2.4
PLU-niosomes 30 : 20	176 ± 6	0.26	–44 ± 1	–5.1
PLU-niosomes 30 : 30	160 ± 7	0.25	–42 ± 1	–4.1
PLU-niosomes 40 : 10	182 ± 5	0.33	–45 ± 1	–4.7
PLU-niosomes 40 : 20	180 ± 3	0.36	–45 ± 2	–8.1
PLU-niosomes 40 : 30	180 ± 4	0.38	–44 ± 2	–9.6
PLU-niosomes 40 : 40	180 ± 7	0.39	–43 ± 2	–7.6
PEC-niosomes 30 : 10	217 ± 1	0.27	–37 ± 2	–6.6
PEC-niosomes 30 : 20	216 ± 4	0.26	–36 ± 3	–2.7
PEC-niosomes 30 : 30	214 ± 4	0.25	–36 ± 1	–4.8
PEC-niosomes 40 : 10	252 ± 11	0.30	–37 ± 2	–4.7
PEC-niosomes 40 : 20	243 ± 6	0.29	–37 ± 2	–6.1
PEC-niosomes 40 : 30	241 ± 3	0.30	–36 ± 3	–4.9
PEC-niosomes 40 : 40	240 ± 2	0.31	–35 ± 3	–5.1

Each value represents the mean ± SD, $n = 6$. $\Delta(BS)_M$, backscattering variation; P90, Phospholipon90G (enriched soy phosphatidylcholine); PLU, Plurol Oleique CC (polyglyceryl-3 dioleate); PEC, Peceol (glycerol monooleate).

6), whereas PEC is a monoolein with a single apolar chain and a little head (HLB = 3). The higher hydrophilicity of PLU in comparison with PEC, along with the presence of cholesterol and lipophilic resveratrol, resulted in the formation of lamellar structures with high curvature grade. As a consequence, all PLU-niosomes were smaller than PEC-niosomes: the average diameter ranged between 160–182 nm for the former, and between 214–252 nm for the latter. Further, the used amount of olein influenced the average size and size distribution of niosomes. Using the highest quantity of PLU or PEC (40 mg/ml), the vesicle average diameter and size distribution increased. Overall, it could be deduced that there was an overlapping effect on vesicle size due to the concomitant presence of olein, cholesterol and resveratrol. Concerning the size distribution of niosomes, PI values were lower than 0.4 in all the formulations, but when the lowest amount of surfactant was used the PI value was < 0.3, indicating an acceptable and always repeatable narrow size distribution.

Cholesterol was used as a membrane stabilizer and its presence was crucial to obtain niosomes, but its amount did not markedly affect vesicle size.

Taking into account these results from niosome characterization, only two formulations for each surfactant were selected to perform further studies. PLU- and PEC-niosomes containing the lowest amount of olein (30 mg/ml) were selected because of their smaller average diameter and narrower size distribution (PI values < 0.3). Since cholesterol was of negligible importance, for each olein only two cholesterol amounts were selected excluding PLU- and PEC-niosomes 30 : 10 that were comparable with the corresponding PLU- and PEC-niosomes 30 : 20. Therefore, all further studies were carried out using liposomes and PLU- and PEC-niosomes 30 : 20 and 30 : 30.

Vesicle stability was evaluated using backscattering variation. An example of backscattering profiles measured using the Turbiscan Lab Expert is shown in Figure 2.

An important backscattering variation at the bottom and the top of the spectra indicates sedimentation and creaming phenomena, respectively, while variations in the middle are linked to vesicle size change. All the niosomal samples exhibited a backscattering decrease (negative variation) never higher than -10%. The backscattering decrease, measured in the middle of the cell ($\Delta(BS)_M$), showed a little variation in the niosomes size, lower than 0.01 μm per day (minimum variation computed by Turbiscan software). Liposomal samples showed a very small positive variation of backscattering, which meant that there was no change in liposome size. The positive sign was probably due to the effect of the particle volume fraction (Φ), because the sedimentation of liposomes leads to a reduction of particle volume fraction in the middle of the sample and it can reach the critical volume fraction (Φc) that corresponds to the concentration at which protons begin to be transmitted, which involves an increase in the backscattering flux.

These results clearly indicated that no irreversible destabilization process occurred, confirming that stable colloidal formulations were obtained.[24, 25]

PLU-niosomes showed sedimentation and creaming phenomena due to the high density of PLU and the low density of cholesterol, respectively, while PEC-niosomes only showed creaming phenomena due to the low density of both compounds. Liposomes showed sedimentation phenomenon. However, since such phenomena are

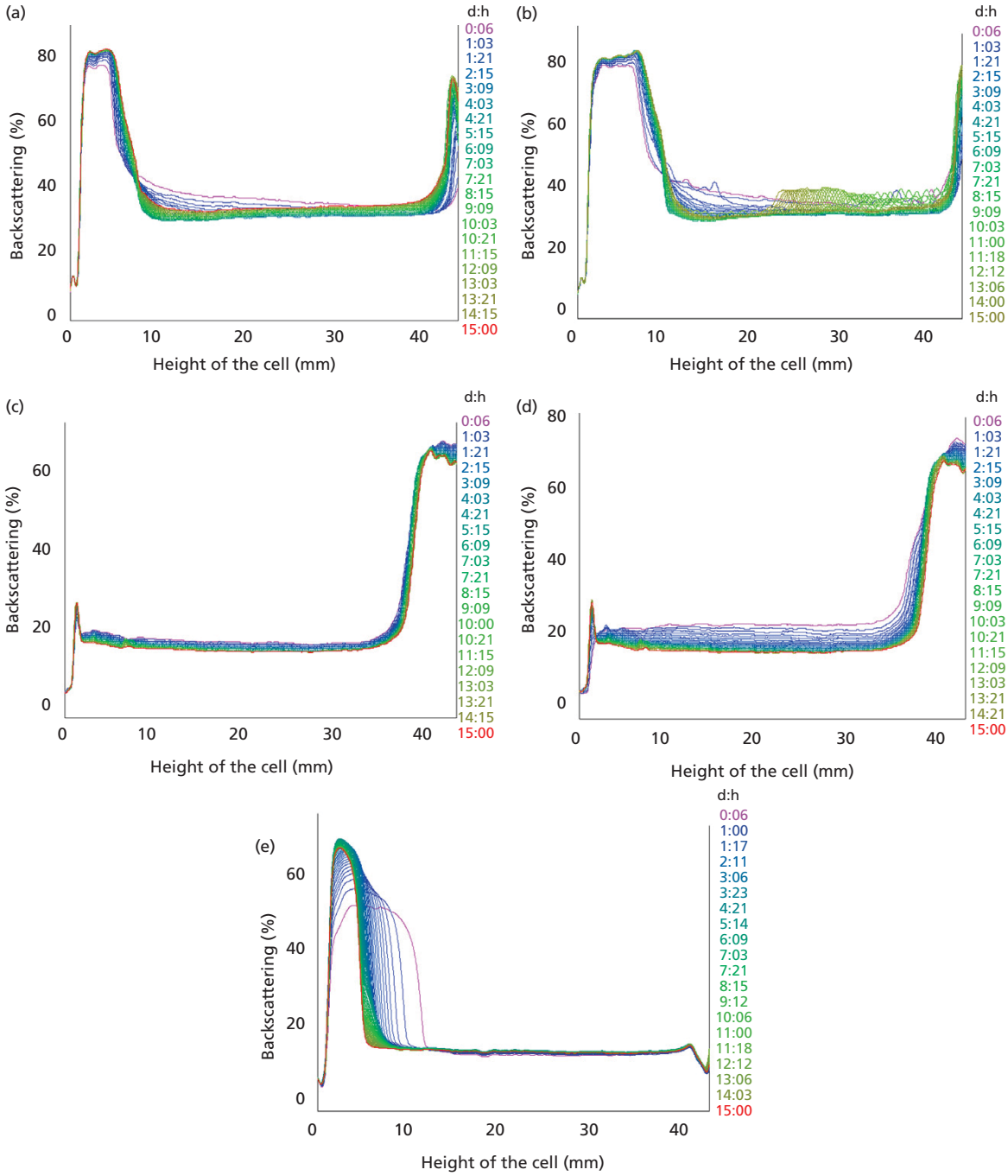

Figure 2 Turbiscan backscattering profiles. (a) Plurol Oleique CC (polyglyceryl-3 dioleate; PLU)-niosomes 30 : 20, (b) PLU-niosomes 30 : 30, (c) Peceol (glycerol monooleate; PEC)-niosomes 30 : 20, (d) PEC-niosomes 30 : 30, (e) Phospholipon90G (enriched soy phosphatidylcholine; P90)-liposomes.

Table 3 Resveratrol entrapment efficiency values and deformation index of niosomal and liposomal systems

Composition	Entrapment efficiency ± SD (%)	Deformation index ± SD
P90-lipsomes	82 ± 2.2	23 ± 1.7
PLU-niosomes 30 : 20	79 ± 2.4	18 ± 3.1
PLU-niosomes 30 : 30	81 ± 3.3	27 ± 4.4
PEC-niosomes 30 : 20	80 ± 2.0	23 ± 4.3
PEC-niosomes 30 : 30	84 ± 1.9	29 ± 3.7

Each value represents the mean ± SD, $n = 6$. P90, Phospholipon90G (enriched soy phosphatidylcholine); PLU, Plurol Oleique CC (polyglyceryl-3 dioleate); PEC, Peceol (glycerol monooleate).

reversible, the system was stable and no significant changes were observed in the niosome size or backscattering profiles with time. So, it can be confirmed that the migration of particles to the bottom and the top of the cell was due to the different densities and not to an irreversible coalescence phenomenon.

Entrapment efficiency

Resveratrol entrapment efficiency values measured for niosomes and liposomes are presented in Table 3. There were no important or statistically significant differences between entrapment efficiency of niosomes and liposomes, or between PLU- and PEC-niosomes. Further, the presence of cholesterol in the vesicles did not improve significantly the entrapment efficiency.

Deformation index determination

To determine the deformability of niosomes and liposomes, deformation index (DI) experiments were carried out using a Liposofast extruder, and the results are shown in Table 3. There were no large differences between niosome or liposome DI value: after extrusion diameter decreased from 10% to 15%. The process was fast and all the loaded volumes were recovered ($J = 1$). PLU- and PEC-niosomes 30 : 30 showed a higher DI than the corresponding PLU- and PEC-niosomes 30 : 20, although these values were too close to be statistically significant ($P > 0.05$). Overall, it could be concluded that all vesicles could be considered deformable, most likely due to the low concentration in lipids. Indeed, in a previous work, lower DI values (up to 7-fold) were obtained for diclofenac-loaded vesicles with a phospholipid content of 90 mg/ml.[22] Several studies have shown that deformable vesicles are more effective than the conventional rigid vesicles in the enhancement of drug transport across the skin. In fact these vesicles, thanks to their high elasticity, are able to easily squeeze out from the stratum corneum reaching deeper skin layers. The mechanism for penetration is the generation of an osmotic gradient due to the evaporation of water while applying the lipid suspension on the skin surface.

Ex-vivo resveratrol distribution and permeation into and through the skin

Skin penetration is the diffusion of a drug across the skin layers into the receptor phase that represents the subcutaneous fluids and blood vessels. Skin penetration involves diffusion across a series of barriers and anatomical structures of the skin through inter- and intra-cellular pathways. To overcome such issues, we attempted to improve resveratrol diffusion using suitable lamellar vesicles, liposomes and niosomes, widely recognized tools for cutaneous enhancement.

To study the influence of vesicles on resveratrol diffusion across the skin, permeation studies were carried out on newborn pig skin. Since human skin is not easily available, many efforts have been made to select a suitable substitute. Although animal skin is different from human skin in several features, it is well known that pig skin is a good substitute in ex-vivo permeation experiments thanks to the similarity of its stratum corneum in terms of lipid composition, even if it presents a marked difference in terms of thickness. Newborn pig stratum corneum is considerably thinner than that of adult pigs, and more similar with that of the human skin, even if the number of hair follicles is higher. Several studies have used newborn pig skin, confirming its suitability in skin permeation screenings (see Manconi *et al.*[22] and references therein).

To get a cutaneous effect of resveratrol, an accumulation of the drug in the deeper skin strata is required. For this reason, the use of drug carriers is recommended. Figure 3 presents the regional distribution of resveratrol in the main layers of the skin (i.e. stratum corneum, epidermis and dermis), as compared with the amount permeated through the skin (receptor fluid mimicking the biological fluids), expressed as the percentage of drug applied onto the skin. As can be observed, the highest amounts of resveratrol were found in the stratum corneum, only a small amount of resveratrol was accumulated into the deeper skin layers (dermis and epidermis). Control liposomes were able to allow drug accumulation into the stratum corneum, showed nil ability to carry and deliver resveratrol to deeper skin layers, and low ability to favour permeation of resveratrol to the receptor fluid. In addition, the amount deposited into the stratum corneum from resveratrol-loaded liposomes was the lowest one, similarly with that obtained with PLU-niosomes 30 : 30 ($P > 0.05$). Therefore, niosomes showed a better behaviour than control liposomes as carriers for resveratrol. In particular, PEC-niosomes 30 : 30 allowed the highest amount of drug to be accumulated in the stratum corneum ($P < 0.01$), 3-fold higher than that of PLU-

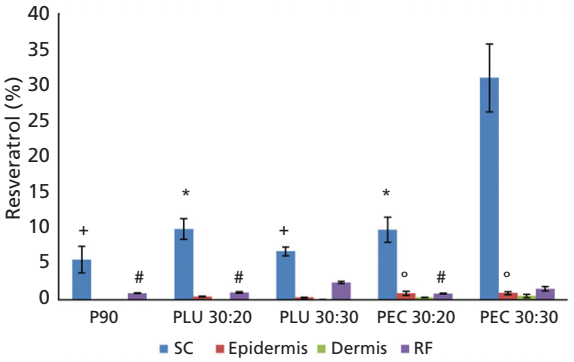

Figure 3 Percentage of resveratrol accumulated in stratum corneum (SC), epidermis, dermis and receptor fluid (RF) referred to as the total amount of resveratrol applied onto the skin into P90-liposomes and PLU- or PEC-niosomes. Each value is the mean ± SD of six experimental determinations. No statistical differences ($P > 0.05$) were found in samples labelled with the same symbol (*, +, #, °). P90, Phospholipon90G (enriched soy phosphatidylcholine); PLU, Plurol Oleique CC (polyglyceryl-3 dioleate); PEC, Peceol (glycerol monooleate).

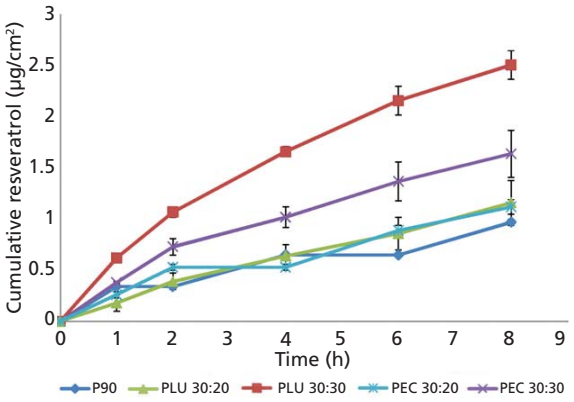

Figure 4 Ex-vivo diffusion of vesicular resveratrol through newborn pig skin (8 h) using P90-liposomes and PLU- or PEC-niosomes. Each value is the mean ± SD of six experimental determinations. P90, Phospholipon90G (enriched soy phosphatidylcholine); PLU, Plurol Oleique CC (polyglyceryl-3 dioleate); PEC, Peceol (glycerol monooleate).

niosomes 30 : 20 and PEC-niosomes 30 : 20, and 4-fold higher than that of PLU-niosomes 30 : 30 and P90-liposomes. Resveratrol loaded into PEC-niosomes was deposited in a higher amount into epidermis and dermis than that provided by PLU-niosomes ($P < 0.05$). The former achieved a resveratrol accumulation of 1.4% in the epidermis and 0.68% in the dermis from the total amount of loaded resveratrol, while the latter achieved only 0.58% in the epidermis, and no resveratrol was found in dermis.

Permeation profiles of resveratrol from liposomes and niosomes were obtained by plotting cumulative amounts of permeated drug (µg/cm^2) against time (Figure 4). As can be seen, permeation curves did not show a classic profile with a steady-state phase. Resveratrol flux was higher in the initial period of the permeation experiments (0–2 h). Afterwards, for all formulations, drug permeation rate was almost constant at all sampling times. Results seemed to indicate that the application of lamellar vesicles (liposomes and niosomes) perturbed the intercorneocytes lamellar spaces, forming channels that allowed a rapid high drug flux through the skin, being the highest when PLU- and PEC-niosomes 30 : 30 were used.

The total amount of resveratrol permeated at 8 h through skin layers was very low in comparison with the total amount of resveratrol deposited onto the skin (Table 4). When resveratrol was formulated in PLU- and PEC-niosomes 30 : 30, the permeation was higher than that obtained using PLU- and PEC-niosomes 30 : 20 and P90-liposomes ($P < 0.05$).

The results obtained during this work seemed to confirm that niosomes were more suitable than liposomes as carriers for cutaneous delivery of resveratrol.

Conclusions

The results underlined that PLU and PEC-niosomes were able to incorporate a high amount of resveratrol (~82%), as well as liposomes, and had comparable physicochemical properties.

Ex-vivo penetration and permeation results from this work have shown that niosomes made from lipophilic surfactant, such as PLU and PEC, were able to deliver resveratrol to the skin to achieve a topical/local effect. In addition, resveratrol permeation through the skin was also provided by niosomes. Niosomes were shown to improve both dermal and transdermal resveratrol delivery in comparison with conventional liposomes.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by the MICINN, Spain, under the grant MICINN-10-CTQ2010-20009-C02-01. Dr D. Pando is grateful for receipt of a graduate fellowship from Severo Ochoa Program (Principado de Asturias, Spain). Dr. C. Caddeo gratefully acknowledges Sardinia Regional

Table 4 Results of permeation experiments from vesicular formulations containing resveratrol

Formulation	Resveratrol permeated (μg/cm² ± SD)		Resveratrol accumulated (μg/cm² ± SD)
	1 h	8 h	
P90-liposomes	0.34 ± 0.01	0.97 ± 0.03	5.85 ± 1.90
PLU-niosomes 30 : 20	0.18 ± 0.08	1.16 ± 0.22	10.39 ± 1.47
PLU-niosomes 30 : 30	0.62 ± 0.01	2.51 ± 0.14	7.28 ± 0.65
PEC-niosomes 30 : 20	0.26 ± 0.03	1.12 ± 0.07	11.92 ± 1.98
PEC-niosomes 30 : 30	0.38 ± 0.01	1.64 ± 0.23	34.88 ± 5.09

Each value represents the mean ± SD, $n = 6$. P90, Phospholipon90G (enriched soy phosphatidylcholine); PLU, Plurol Oleique CC (polyglyceryl-3 dioleate); PEC, Peceol (glycerol monooleate).

Government for the financial support (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007–2013 – Axis IV Human Resources, Objective l.3, Line of Activity l.3.1 'Avviso di chiamata per il finanziamento di Assegni di Ricerca').

Acknowledgements

Sardegna Ricerche Scientific Park (Pula, CA, Italy) is acknowledged for free access to facilities of the Nanobiotechnology Laboratory.

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III. Resveratrol entrapped niosomes as yoghurt additive

The aim of this work was to formulate resveratrol (RSV) entrapped niosomes for oral administration, which could be used as yoghurt additive. For niosomes preparation, a slightly modified thin film hydration method was selected. Dodecanol was used as membrane stabilizer, instead of cholesterol, to avoid adverse health effects and the potential rejection by consumer of final product. It was previously reported that stable niosomes could be prepared using fatty alcohols.

The key parameters involved in niosomes preparation, such as surfactant to dodecanol weight ratio and agitation speed, were optimised using a factorial design of experiments and a statistical analysis of variance (ANOVA). This enabled to assess each parameter contribution to mean size, polydispersity index (PDI), and entrapment efficiency (EE) of niosomes. The textural properties of RSV enriched yoghurt were also analysed.

Article 3. D. Pando, M. Beltrán, I. Gerone, M. Matos, C. Pazos. *Resveratrol entrapped niosomes as yoghurt additive*.

Food Chemistry 170 (2015) 281-287. DOI:10.1016/j.foodchem.2014.08.082

Personal contribution to work

I undertook the experimental work in cooperation with Miriam Beltrán and Ilay Gerone. I also conducted the factorial experimental design and was responsible for developing some analytical techniques, such as Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Finally, I prepared the first draft of the manuscript and figures included under the supervision of Dr. María Matos and Prof. Carmen Pazos.

III. Niosomas conteniendo resveratrol como aditivos para yogur

El objetivo de este trabajo era formular niosomas conteniendo resveratrol (RSV) para uso oral, que pudieran utilizarse como aditivos en la fabricación de yogures funcionales. Para su preparación, se seleccionó el método de hidratación de película, ligeramente modificado. Como estabilizante de membrana, se utilizó dodecanol en lugar de colesterol, buscando evitar los efectos perjudiciales para la salud de este último, así como el posible rechazo de los consumidores al producto de consumo final. La información disponible en la bibliografía indicaba que resultaba factible preparar niosomas estables utilizando alcoholes grasos.

Se optimizaron los parámetros más importantes involucrados en la preparación de niosomas, como relación en peso tensioactivo – dodecanol y velocidad de agitación, utilizando un diseño factorial de experimentos y un análisis de la varianza (ANOVA). De esta forma, se estudió la contribución de cada parámetro sobre el tamaño medio, el índice de polidispersidad (PDI) y la eficacia de encapsulación (EE) de los niosomas. Asimismo, se analizaron las propiedades texturales de los yogures enriquecidos con RSV.

Artículo 3. D. Pando, M. Beltrán, I. Gerone, M. Matos, C. Pazos. *Resveratrol entrapped niosomes as yoghurt additive*

Food Chemistry 170 (2015) 281-287. DOI:10.1016/j.foodchem.2014.08.082

Contribución personal al trabajo

El trabajo experimental lo llevé a cabo en colaboración con Miriam Beltrán e Ilay Gerone. Asimismo, realicé el diseñó de experimentos y puse a punto las técnicas analíticas empleadas, caso de la cromatografía líquida de alta resolución en fase inversa (RP-HPLC). Finalmente, redacté el borrador del manuscrito y elaboré las figuras en él incluidas, con la supervisión de mis directoras de Tesis, Dra. María Matos y Prof. Carmen Pazos.

Food Chemistry 170 (2015) 281–287

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Resveratrol entrapped niosomes as yoghurt additive

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ARTICLE INFO

Article history:
Received 11 April 2014
Received in revised form 7 July 2014
Accepted 14 August 2014
Available online 24 August 2014

Keywords:
Niosome
Resveratrol
Functional food
Yoghurt
Factorial design
ANOVA

ABSTRACT

Nanodesign of niosomes containing resveratrol (RSV) was carried out using food-grade surfactants with dodecanol to stabilise the membrane. Niosomes were prepared using a modified thin film hydration method.

A factorial design analysis was carried out to reduce the number of experiments. The response factors were: mean size, polydispersity index (PDI) and entrapment efficiency (EE). Agitation speed and surfactant to dodecanol weight ratio were selected as key parameters for niosomes preparation. Parameter contribution was determined using a statistical analysis of variance (ANOVA).

Niosomes formulated with Span 60 or Maisine 35-1 as surfactants, and dodecanol as stabiliser, were able to incorporate RSV. These niosomes exhibited a small mean size, narrow size distribution, high RSV entrapment efficiency and good stability. RSV addition did not involve changes in the textural properties of regular yoghurt demonstrating that RSV entrapped niosomes are suitable additives in these dairy products.

1. Introduction

Natural products have been widely used to prevent or mitigate various diseases and lately there has been a growing interest in research, development and commercialisation of functional foods, nutraceuticals and dietary supplements (Dewapriya & Kim, 2014; Ortuño et al., 2010; Shahidi, 2009). The term "*nutraceutical*" is defined as any food-based substance that provides health benefits, including prevention and treatment of diseases (Defelice, 1995). This term is commonly used in marketing although there is no regulatory definition, and in certain countries the terms functional food and nutraceutical are used interchangeably. In all cases, the main focus is to improve health and reduce the risk of diseases mainly through prevention (Shahidi, 2009). Moreover, pharmaceutical and biotechnology companies have made significant investments in the discovery and production of nutraceuticals/functional foods (Kalra, 2003; Nelson, 1999).

Resveratrol (RSV) is a natural polyphenol which can be considered as a *nutraceutical* because of benefits such as anticancer activity (Jang & Surh, 2003; Surh et al., 1999), lifespan extension (Howitz et al., 2003), cardioprotection (Hung, Chen, Huang, Lee, & Su, 2000), antioxidant activity (Frankel, Waterhouse, & Kinsella, 1993; Fremont, Belguendouz, & Delpal, 1999), inhibition of platelet aggregation (Bertelli et al., 1995; Chung, Teng, Cheng, Ko, & Lin, 1992) and antiinflammatory activity (Pace-Asciak, Hahn, Diamandis, Soleas, & Goldberg, 1995). Consequently, RSV may prove to be a useful ingredient for functional foods.

RSV is a photosensitive molecule that exists in *cis* and *trans* structural *isomers*, but only *trans*-RSV demonstrates health benefits. The change from the active *trans* isomer to the inactive *cis* isomer is mainly caused by exposure to light. RSV therefore needs to be encapsulated. Encapsulation facilitates control of the rate of RSV release and protects the molecule during digestion, from degradation under pancreatic conditions (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). It can also help to mask undesired flavours.

RSV encapsulation studies have been done in pharmaceutical and cosmetic industries with the aims of preventing degradation, increasing its solubility in water, and targeting specific locations in the body by using multiparticulate forms and colloidal carriers (Amri, Chaumeil, Sfar, & Charrueau, 2012; Caddeo, Teskač, Sinico, & Kristl, 2008; Donsi, Sessa, Mediouni, Mgaidi, & Ferrari, 2011; Kristl, Teskač, Caddeo, Abramovic, & Sentjurc, 2009; Lucas-Abellán, Fortea, López-Nicolás, & Núñez-Delicado, 2007; Matos, Gutiérrez, Coca, & Pazos, 2014; Pando, Caddeo, Manconi, Fadda, & Pazos, 2013; Pando, Gutiérrez, Coca, & Pazos, 2013; Peng et al., 2010; Sessa et al., 2014; Teskač & Kristl, 2010; Wang et al., 2011).

Niosomes are vesicles formed by the self-assembly of non-ionic surfactants in aqueous media resulting in closed bilayer structures

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http://dx.doi.org/10.1016/j.foodchem.2014.08.082
0308-8146/

(Uchegbu & Vyas, 1998). These vesicles are commonly used to encapsulate both hydrophilic and lipophilic compounds, for either food, pharmaceutical or cosmetic applications. Hydrophilic compounds are entrapped in the aqueous compartments between the bilayers while the lipophilic components are preferentially located within the surfactant bilayer (Devaraj et al., 2002).

Surfactants are versatile products of the chemical industry, and the large number of available non-ionic surfactants enables the design of niosomes for specific applications (Manosroi et al., 2003). The main advantage of niosomes, with respect to other encapsulation technologies, such as liposomes (Fang & Bhandari, 2010; Gibis, Zeeb, & Weiss, 2014), is their low cost, high stability, and biocompatibility (Kopermsub, Mayen, & Warin, 2011).

Size distribution, stability and entrapment efficiency are the key parameters to obtain optimal niosomal systems. Two interacting variables involved in niosomes preparation (*i.e.* agitation speed and surfactant to stabiliser weight ratio) have to be tested to properly analyse the behaviour of the system and to optimise system parameters. Changing one factor at a time is not an efficient and economic strategy because it does not provide information regarding the optimum location and it does not take into account interactions of parameters. Factorial design and analysis of variance (ANOVA) methodology are appropriate and efficient statistical tools, which make it possible to study the effects of several factors that influence responses by varying them simultaneously within a limited number of experiments (Kincl, Turk, & Vrečer, 2005; Martínez-Sancho, Herrero-Vanrell, & Negro, 2004).

The aim of this work is to formulate RSV entrapped niosomes for oral administration, using a modified thin film hydration method. Dodecanol was selected as a membrane stabiliser, because it has previously been reported that stable niosomes can be prepared with fatty alcohols (Devaraj et al., 2002), instead of cholesterol, which avoids the gel–liquid phase transition of niosomes. By contrast cholesterol may not be suitable for use in functional foods because of potential adverse health effects.

The key parameters involved in niosome preparation (*i.e.* surfactant to dodecanol weight ratio and agitation speed) were optimised by a factorial design of experiments and statistical analysis of variance (ANOVA) to assess their contributions to mean size, polydispersity index (PDI), and entrapment efficiency (EE). Finally, the best formulation for each surfactant was selected for subsequent preparation of yoghurts enriched with RSV. The textural properties of these yoghurts were also analysed.

2. Materials and methods

2.1. Materials

Trans-resveratrol (RSV), with a purity >99%, sorbitan monostearate (Span 60, S60), lauryl alcohol (dodecanol, Dod) and absolute ethanol were supplied by Sigma–Aldrich (USA). Labrasol (Lab) and Maisine 35-1 (Mai) were a gift from Gattefossé (France). Methanol, acetonitrile, 2-propanol and acetic acid of HPLC-grade were obtained from Sigma–Aldrich (USA). Deionised water was used in all experiments.

2.2. Preparation of niosomes

Niosomes were prepared by the thin film hydration method (Baillie, Florence, Hume, Muirhead, & Rogerson, 1985; Bangham, Standish, & Watkins, 1965) with minor modifications, followed by agitation–sonication (Pando, Gutiérrez, et al., 2013). Accurately weighed amounts of surfactant (S60, Lab or Mai) and dodecanol in different weight ratios, from 1:0.5 to 1:1.5, were dissolved in 20 ml of a solution of ethanol containing a known concentration of RSV and placed into a 100 ml round bottom flask. Ethanol was then removed at 40 °C under reduced pressure in a rotary evaporator (Buchi, Switzerland). The dried film was hydrated with 40 ml of deionised water at 60 °C to achieve a RSV concentration of 150 mg/L. The resulting sample was subsequently homogenised (SilentCrusher M, rotor model 22G, Heidolph, Germany) at speeds ranging from 5000 to 15,000 rpm depending on the experiment, and further sonicated for 30 min (CY-500 sonicator, Optic Ivymen System, Spain), using 45% amplitude, 500 W power and 20 kHz frequency.

2.3. Characterisation of RSV entrapped niosomes

2.3.1. Vesicle size and zeta potential measurements

Mean (Z-Average) sizes and PDI of the niosomes were determined via Dynamic Light Scattering (DSL) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Three independent samples were taken from each formulation, and each was measured three times at room temperature without dilution.

For determination of zeta potentials (ζ-potential), three independent samples were also taken from each formulation and measure three times at room temperature. Each of them were diluted (1:10 v/v) before measuring vesicle electrophoretic mobility using M3-PALS (Phase Analysis Light Scattering) technique. High absolute values of ζ-potential indicate electrostatic repulsion between vesicles. Such conditions are linked to high stability.

2.3.2. Stability measurements

Stability of the niosomes was determined by measuring backscattering (BS) profiles in a Turbiscan Lab® Expert apparatus (Formulaction, France) provided with an Ageing Station (Formulaction, France). Undiluted niosome samples were placed in cylindrical glass test cells and the backscattered light was monitored as a function of time and cell height for 15 days, every 3 h, at 30 °C. The optical reading head scans the sample in the cell, providing BS data every 40 μm in % relative to standards (suspension of monodisperse spheres and silicone oil) as a function of the sample height (in mm). These profiles provide a macroscopic fingerprint of the niosomes at a given time, providing useful information about changes in vesicle size distribution and/or appearance of a creaming layer or a clarification front with time (Pando, Caddeo, et al., 2013; Pando, Gutiérrez, et al., 2013).

2.4. RSV entrapment efficiency (EE)

Entrapped RSV was separated from free RSV by dialysis. Samples (2 ml) were placed into a dialysis bag, which was immersed in 1000 ml of deionised water at room temperature and stirred at 500 rpm for 2 h. Dialysed and non-dialysed samples were diluted 1:10 (v/v) with methanol to rupture the vesicle membranes enabling extraction of RSV. Later, RSV concentration was determined by chromatography (HP series 1100 chromatograph, Hewlett Packard, USA). The system was equipped with a UV/VIS absorbance detector HP G1315A and a fluorescence detector 1260 Infinity A (Agilent Technologies, USA). A wavelength of 305 nm was used for the UV/VIS detector while fluorescence detector used 310/410 nm of $\lambda_{excitation}/\lambda_{emission}$. The analytical column was a Zorbax Eclipse Plus C_{18} of 5 μm particle size, 4.6 mm × 150 mm (Agilent Technologies, USA).

The mobile phase consisted of a mixture of (A) 100% milliQ-water and (B) 100% methanol with gradient elution at a flow rate of 0.8 ml/min. The step gradient started with a mobile phase of 80% (A) running 100% mobile phase (B) after 5 min for 10 min. The mobile phase (B) was fed for 2 min after each injection to prepare the column for the next sample. The separation was carried out at 30 °C.

2.5. Preparation of yoghurt enriched with RSV

To prepare yoghurt enriched with RSV, 10.6 ml of the niosomal suspension containing RSV was placed into a beaker and diluted to 200 ml with a mixture of fresh pasteurised milk and natural yoghurt (8:1 v/v).

The sample was incubated for 12 h at 40 °C in a yoghurt maker, model OU-YG01 (Moulinex, France). Once the yoghurt was formed, it was cooled and stored at 4 °C in a refrigerator for at least 2 h.

2.6. Textural analysis of yoghurt enriched with RSV

A textural analysis was carried out both for regular yoghurt and yoghurt containing RSV to study possible changes resulting from the addition of RSV entrapped niosomes.

The firmness and adhesiveness of both types of yoghurt were measured with a TA.XTplus texture analyser (Stable Micro Systems, USA) using a single compression cycle test. An SMSP/0.5S probe was employed using a penetration speed of 2 mm/s and a penetration depth of 5 mm. The test was carried out immediately after removing the sample from refrigeration at 4 °C.

Table 1
Experimental design schedule for the L_9 array.

Trial	Agitation speed (rpm)	Surfactant:dodecanol ratio (w/w)
1	5000	1:0.5
2	5000	1:1
3	5000	1:1.5
4	10,000	1:0.5
5	10,000	1:1
6	10,000	1:1.5
7	15,000	1:0.5
8	15,000	1:1
9	15,000	1:1.5

Firmness was defined as the force necessary to reach the maximum depth and adhesiveness was calculated as the negative force area of the cycle, representing the work necessary to pull the compressing plunger away from the sample (Bourne, 2002).

2.7. Statistical analysis

All data were expressed as the mean ± SD (standard deviation) of three independent experiments and statistical analysis of variance (ANOVA) was applied. Fisher's test ($p < 0.05$) was used to calculate the least significance difference (LSD) using statistical software (Microsoft Excel 2010).

3. Results and discussion

3.1. Experimental design

Niosomes were prepared at three different agitation speeds (5000, 10,000 and 15,000 rpm) and surfactant:dodecanol weight ratio values of 1:0.5, 1:1 and 1:1.5. The objective was to study, using ANOVA statistical analysis, how these key factors affected the response parameters: mean size, PDI and EE. Factors and levels were combined according to the orthogonal array L_9 indicated in Table 1.

Dispersions were characterised to determine the best formulation and method of preparation for each surfactant. The best formulations were then selected for the subsequent preparation of yoghurt enriched with RSV.

3.1.1. Mean size

Table 2 shows mean sizes, PDI and EE values of the niosomes obtained for the three surfactants operating at different agitation speeds and surfactant:dodecanol weight ratios.

Table 2
Experimental design results for niosome mean size, PDI and EE as response parameters.

Trial	Size (nm)			PDI			EE (%)		
	S60-Dod	Lab-Dod	Mai-Dod	S60-Dod	Lab-Dod	Mai-Dod	S60-Dod	Lab-Dod	Mai-Dod
1	205	333	237	0.342	0.251	0.386	29.0	59.1	37.0
	220	351	216	0.326	0.276	0.282	29.3	51.8	25.4
	222	401	221	0.333	0.240	0.302	28.3	55.5	27.9
2	139	294	272	0.269	0.229	0.305	33.7	46.7	37.4
	162	233	296	0.241	0.129	0.488	52.0	52.7	36.9
	163	329	368	0.236	0.217	0.426	41.1	52.6	41.5
3	196	368	331	0.352	0.332	0.357	52.0	16.3	44.9
	175	543	269	0.289	0.556	0.321	47.4	24.5	27.0
	183	412	302	0.331	0.452	0.392	43.2	19.7	31.1
4	163	427	223	0.204	0.403	0.367	22.9	61.7	39.6
	161	344	277	0.215	0.349	0.410	21.5	63.9	25.1
	171	429	254	0.233	0.365	0.395	23.0	60.2	30.1
5	185	314	213	0.271	0.281	0.253	40.5	30.3	48.5
	188	389	224	0.272	0.412	0.232	40.0	48.6	35.1
	196	285	230	0.259	0.344	0.271	36.7	33.6	39.6
6	178	378	311	0.261	0.501	0.429	56.8	27.7	55.1
	183	350	252	0.270	0.448	0.302	58.5	25.5	49.7
	191	454	295	0.251	0.403	0.365	61.1	32.4	56.0
7	162	418	211	0.255	0.403	0.232	22.5	50.0	39.2
	195	379	222	0.261	0.379	0.292	16.8	34.3	29.9
	218	302	216	0.219	0.421	0.257	26.1	41.9	32.6
8	227	373	203	0.235	0.364	0.265	59.1	41.4	37.3
	190	544	164	0.271	0.490	0.249	53.2	33.6	47.3
	178	465	205	0.346	0.431	0.218	49.3	42.8	42.1
9	165	688	170	0.267	0.481	0.241	58.5	31.8	56.4
	167	408	166	0.248	0.582	0.241	72.5	22.8	58.6
	173	586	191	0.225	0.472	0.211	60.3	35.9	43.4

The results in Table 2 exhibit a clear relationship between the type of surfactant used and the resulting mean size of the niosome ($p < 0.05$), for all experimental conditions tested. The smallest mean size corresponded to S60 niosomes, while Lab niosomes showed the highest values for this response factor. The niosomes formulated with S60 had mean sizes between 139 and 227 nm with an average value of 183 nm, while the Mai-niosome mean size ranged from 164 to 368 nm, and an average value of 242 nm. These two surfactants have similar molecular structures, with the same number of carbon atoms (17) for the alkyl chain, but differing in the hydrophilic portion with sorbitan monosterate for S60, and glycerol monosterate for Mai. Consequently, their hydrophilic–lipophilic balance (HLB) is quite similar, being 4.7 and 4.0 for S60 and Mai, respectively. Thus, it was expected that niosomes formulated with these surfactants should be similar in size, as was observed experimentally.

Moreover, when Mai was used as a surfactant, a close correlation between the agitation speed and niosome mean size ($p < 0.05$) was observed; smaller niosome mean sizes were produced at higher agitation speeds as was previously reported (Pando, Gutiérrez, et al., 2013). No such influence was observed when S60 or Lab was used.

Changes in niosome mean size resulting from different surfactant:dodecanol weight ratios were also observed when Lab was used as a surfactant ($p < 0.05$). This result may be attributed to its large HLB value (14), which results in a greater affinity for the aqueous phase, thereby preventing vesicle formation in the absence of a stabiliser. This situation leads to a close link between the surfactant:dodecanol weight ratio and mean size.

For niosomes formulated with Lab, the smallest mean size was obtained at a surfactant:dodecanol weight ratio of 1:0.5. However, dodecanol did not stabilise the niosomal structure well. The Lab-dodecanol system began to coagulate, leading to larger vesicle sizes and phase separation.

3.1.2. Polydispersity index (PDI)

PDI values of niosome size distributions for the three surfactants are also shown in Table 2.

The PDI results exhibit a clear ($p < 0.05$) dependence on the type of surfactant. Niosomes with the smallest PDI were obtained with the S60 surfactant, followed by those using Mai and Lab. Niosomes prepared with S60 led to PDIs in the range of 0.204–0.352, 0.279 being the average value. The PDI of niosomes formulated using Mai ranged from 0.211 to 0.488, with an average value of 0.314. Narrower distributions were obtained with these two surfactants, since niosomes obtained from formulations containing Lab led to PDI values in the range of 0.129–0.582 with an average value of 0.378, which was too close to the limit value of 0.4. Once this value is exceeded, the corresponding PDI values are no longer considered narrow.

All the surfactants resulted in a significant PDI dependence on the agitation speed ($p < 0.05$). For formulations containing S60 and Mai, lower PDI values were obtained at higher agitation speeds, while for niosomes prepared using Lab, lower agitation speeds were associated with lower PDI values.

Table 3
Optimal key parameters for preparation of RSV entrapped niosomes.

Niosomal system	Agitation speed (rpm)	Surf:Dod ratio (w/w)	Size (nm)	PDI	EE (%)
S60-Dod	15,000	1:1.5	168 ± 4	0.247 ± 0.018	63.8 ± 7.6
Lab-Dod	5000	1:0.5	362 ± 35	0.255 ± 0.021	55.4 ± 3.7
Mai-Dod	15,000	1:1.5	175 ± 13	0.260 ± 0.025	52.8 ± 8.2

As was stated earlier, only the Lab surfactant showed a close relationship between the surfactant:dodecanol weight ratio, and the mean size and PDI of the niosomal system. For the Lab-Dod niosomes, the lowest PDI value was obtained for a surfactant:dodecanol weight ratio of 1:0.5, in accordance with the lowest niosome mean size.

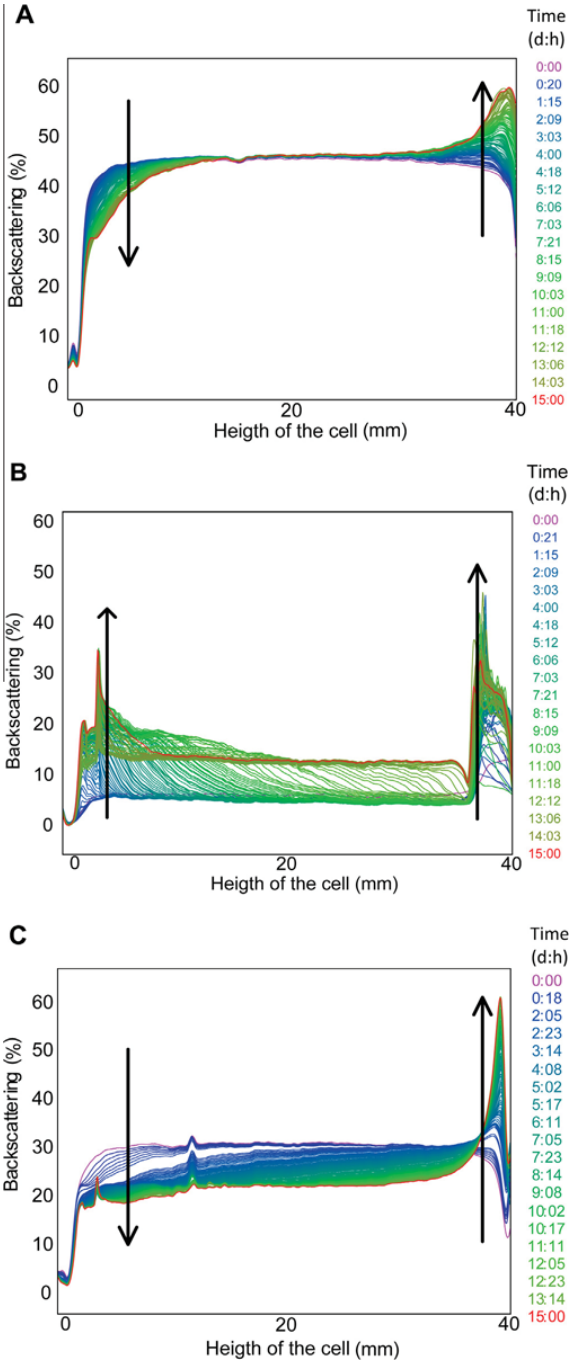

Fig. 1. Backscattering profiles of RSV entrapped niosomes made of: (A) S60 and dodecanol (1:1.5 w/w) at 15,000 rpm; (B) Lab and dodecanol (1:0.5 w/w) at 5000 rpm; (C) Mai and dodecanol (1:1.5 w/w) at 15,000 rpm.

3.1.3. RSV entrapment efficiency (EE)

The EE was strongly dependent on the surfactant used and surfactant:dodecanol weight ratio ($p < 0.05$), while agitation speed had no effect ($p > 0.05$).

For niosomes prepared with S60, EE values ranged between 16.8% and 72.5%, with an average value of 42.0%, while for niosomes prepared with Lab, the range of EE was 16.3–63.9%, with an average value of 40.6%. The lowest EE values were obtained with Mai (25.1–58.6%).

With respect to RSV entrapment efficiency, each surfactant exhibited a different behaviour at the various surfactant:dodecanol weight ratios tested. Niosomes prepared with S60 and Mai led to higher EE values at higher surfactant:dodecanol weight ratio (1:1.5), while for niosomes formulated with Lab, higher EE values were obtained at the lowest surfactant:dodecanol weight ratio (1:0.5). These findings can be explained in terms of similar HLB values for two of the surfactants (HLB = 4.7 and 4.0 for S60 and Mai respectively) whereas Lab is much more hydrophilic (HLB = 14).

3.1.4. Optimal operating conditions

For S60-Dod niosomes, the best results were obtained when agitated at 15,000 rpm, corresponding to the lowest PDI value, for a 1:1.5 surfactant:dodecanol weight ratio.

However, for the Lab-Dod niosomes the lowest PDI value was obtained at the lowest agitation speed (5000 rpm) and lowest surfactant:dodecanol weight ratio (1:0.5). These conditions lead to a smaller mean size and a higher EE value.

Finally, for Mai-Dod niosomes, lower PDI values were observed at higher agitation speeds (15,000 rpm). Higher surfactant:dodecanol weight ratios led to smaller mean sizes.

A summary of the optimal key parameters is displayed in Table 3.

3.2. Niosome stability

The stability of the niosomes was determined by the variation of the BS (ΔBS) over time. Changes in niosome size are directly related to the ΔBS values measured in the middle zone of the Turbiscan cell (ΔBS_M). An increase in niosome size indicates that coagulation and/or flocculation have taken place, leading to system destabilisation. In addition, BS variations at the bottom (ΔBS_B) and top (ΔBS_T) of the measuring cell are linked to sedimentation and creaming phenomena respectively. Turbiscan equipment has been widely used to select the best formulation for colloidal systems employed in food applications (Matos et al., 2014; Márquez, Palazolo & Wagner, 2007; Pando, Caddeo, et al., 2013; Pando, Gutiérrez, et al., 2013).

The ζ-potential is strongly linked to stability of the niosomes. High absolute values of ζ-potential imply a repulsive force between niosomes with a concomitant increase in stability. On the other

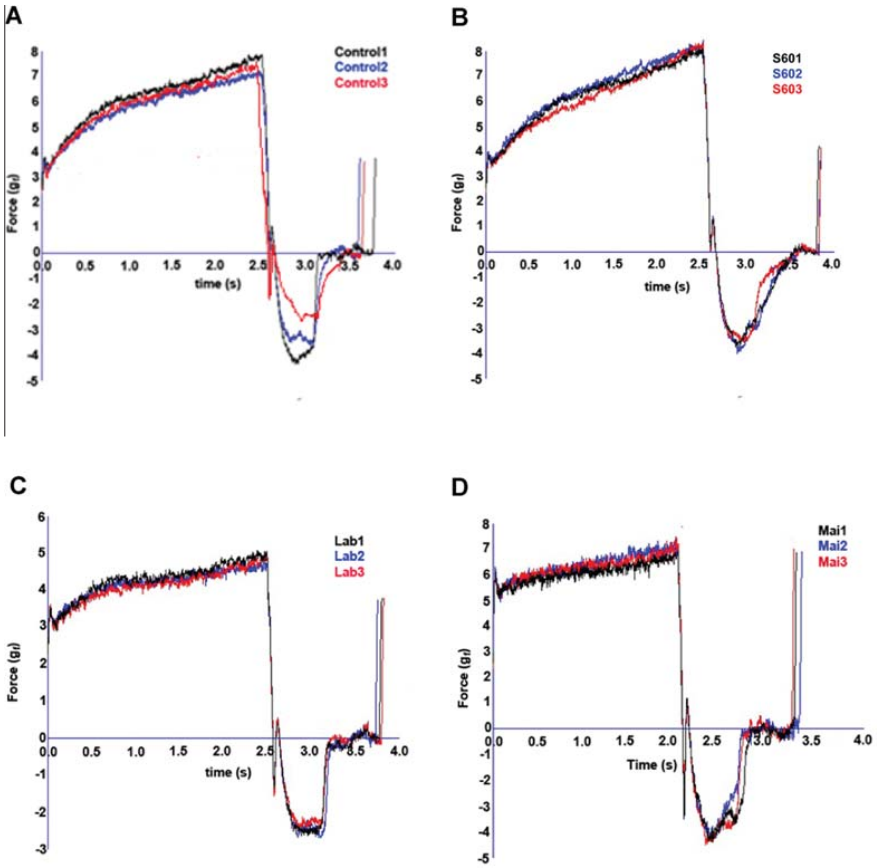

Fig. 2. Textural properties of yoghurt: (A) regular yoghurt, no additives; (B) yoghurt with RSV entrapped S60-Dod niosomes; (C) yoghurt with RSV entrapped Lab-Dod niosomes; (D) yoghurt with RSV entrapped Mai-Dod niosomes.

Table 4
Firmness and adhesiveness values of different yoghurts.

System	Firmness (g_F)	Adhesiveness (g_F)
Regular yoghurt (control)	7.535 ± 0.321	−4.035 ± 0.518
RSV S60-Dod yoghurt	7.642 ± 0.106	−3.538 ± 0.173
RSV Lab-Dod yoghurt	5.005 ± 0.105	−2.615 ± 0.085
RSV Mai-Dod yoghurt	7.186 ± 0.124	−4.166 ± 0.055

hand, close to zero or even low absolute values, lead to unstable systems with resultant coagulation or flocculation processes.

The stability of samples from the best formulations was monitored every 3 h for 15 days.

S60-Dod niosomes prepared at 15,000 rpm with a surfactant:dodecanol weight ratio of 1:1.5 were highly stable with no changes in niosome size, although a slight variation in BS values was observed. Thus, the ΔBS_M values were quite low and destabilisation processes, such as coagulation or flocculation, did not occur. However, a creaming phenomenon was observed by an increase in BS at the top of the cell (ΔBS_T), due to particle accumulation in this zone and clarification at the bottom of the cell. These phenomena were accompanied by a corresponding decrease in BS (ΔBS_B) (Fig. 1A). Creaming can be attributed to the presence of free dodecanol molecules, which did not form niosomal bilayer but instead migrated towards the top of the cell because of their lower density (850 kg/m^3).

Moreover, this niosomal system was characterised by a ζ-potential value of −50.1 ± 0.8 mV. This result agreed well with the high stability observed via Turbiscan measurements, confirming the absence of coagulation/flocculation processes resulting from electrostatic repulsion effects among the niosomes.

Lab-Dod niosomes prepared at 5000 rpm with a surfactant:dodecanol weight ratio of 1:0.5 showed lower stability over time. A sharp variation in BS values was observed in the middle zone of the cell (Fig. 1B), which indicated a significant change in niosome size. A creaming layer also appeared at the top of the cell leading to an increase in ΔBS_T and a simultaneous increase of ΔBS_B caused by sedimentation phenomena.

This niosomal system had a ζ-potential of −18.7 ± 0.6 mV. Thus, electrostatic repulsion would be weaker, making an increase in niosome size more likely.

Niosomes prepared at 15,000 rpm using Mai-Dod with a surfactant:dodecanol weight ratio of 1:1.5 exhibited acceptable stability. An increase in mean size of the niosomes, resulting from the BS decrease in the middle zone of the cell (ΔBS_M) was observed. In addition, a creaming phenomenon appeared due to free dodecanol molecules with the corresponding decrease in ΔBS_B, and ΔBS_T increase (Fig. 1C). This system had a ζ-potential of −39.9 ± 0.3 mV corresponding to the acceptable stability of these niosomes.

The best formulation of the RSV entrapped niosomes was obtained using S60-Dod. This niosomal system demonstrated much better stability than niosomes formulated with Lab-Dod.

3.3. Textural analysis of yoghurt enriched with niosomes containing resveratrol

The textural analysis of regular yoghurt and yoghurt enriched with RSV, by adding niosomes prepared with S60, Lab and Mai, was carried out in order to ascertain if any textural difference caused by addition of niosomes was present.

Fig. 2 shows the textural analysis for each sample obtained by measuring the force applied on yoghurt surface as a function of time.

Values of the textural parameters studied (firmness and adhesiveness) are summarised in Table 4.

Only the yoghurt containing RSV Lab-Dod niosomes showed significant differences in firmness with respect to the control regular yoghurt.

The yoghurt containing RSV Mai-Dod niosomes exhibited adhesiveness closer to that of the control, followed by yoghurt enriched with RSV S60-Dod niosomes. By contrast, the addition of RSV Lab-Dod niosomes to yoghurt led to significant decrease in adhesiveness.

Thus, yoghurts with RSV entrapped niosomes formulated with S60 or Mai demonstrated textural properties similar to those of the control, but the yoghurt enriched with Lab niosomes containing RSV led to textural properties slightly different, which could be readily apparent in the final product.

4. Conclusions

Combination of factorial experimental design with statistical analysis of variance (ANOVA) is a good methodology to employ in ascertaining the best formulation for niosomal systems entrapping RSV, within a moderate number of experiments.

Dodecanol could replace cholesterol as stabiliser in formulations of food-grade niosomes.

Niosomes formulated with Span 60 or Maisine 35-1 as surfactants and dodecanol as stabiliser are successful preparations for incorporation of RSV. These niosomes exhibit a small mean size, narrow size distribution, high RSV entrapment efficiency, and good stability.

Addition of RSV did not involve changes in the textural properties of regular yoghurt demonstrating that RSV entrapped niosomes are suitable additives in these dairy products.

Acknowledgements

This work was supported by the Ministerio de Ciencia e Innovación (MICINN, Spain), under the Grant MICINN-10-CTQ2010-20009-C02-01. This study was co-financed by the Consejería de Educación y Ciencia del Principado de Asturias (Ref. FC-04-COF-50-MEC and PCTI Asturias 2006–2009, Ref. EQP06-024).

D. Pando acknowledges receipt of a graduate fellowship from Severo Ochoa Program (Principado de Asturias, Spain).

The authors thank Prof. Coca (University of Oviedo) and Prof. Hill (University of Wisconsin) for fruitful discussions, and A. González for her kind help with lab work.

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IV. Formulation of resveratrol entrapped niosomes for topical use

The aim of this work was to evaluate and compare two different preparation techniques of resveratrol (RSV) entrapped niosomes for topical use. Additionally, the ex-vivo RSV percutaneous absorption in this type of innovative vesicles was also evaluated.

The *thin film hydration*, with minor modifications, followed by *sonication* (TFH-S), and *ethanol injection*, also slightly modified (EIM), were the methods selected for niosomes preparation.

Niosomes were formulated using Gelot 64 (G64) as surfactant and two suitable skin-compatible unsaturated fatty acids, oleic (OA) and linoleic acid (LA), commonly used in pharmaceutical formulations as penetration enhancers,. All niosomes prepared were characterized by measuring the following parameters: mean size and particle size distribution, stability, and entrapment efficiency (EE).

Article 4. D. Pando, M. Matos, G. Gutiérrez, C. Pazos. ***Formulation of resveratrol entrapped niosomes for topical use***.

Colloids and Surfaces B: Biointerfaces. Under revision

Personal contribution to work

Based on the experience acquired during my stay at University of Cagliari, I conducted the ex-vivo transdermal experiments on newborn pig skin using Franz diffusion cells with the help of the laboratory technician, Andrea González. I also performed the factorial design of experiments and analysis of variance (ANOVA). Finally, I prepared the first draft of the manuscript in collaboration with Dr. Gemma Gutiérrez, and under the supervision of Dr. María Matos and Prof. Carmen Pazos.

IV. Formulación de niosomas conteniendo resveratrol para uso tópico

El objetivo de este estudio fue llevar a cabo un análisis comparativo entre dos métodos diferentes para la preparación de niosomas conteniendo resveratrol (RSV) de uso tópica. Asimismo, se evaluó la capacidad de absorción percutánea del RSV en estas novedosas vesículas.

Los dos métodos seleccionados fueron el de *hidratación de película*, ligeramente modificado, seguido de *sonicación* (TFH-S) y el de *inyección de etanol* (EIM), también con pequeñas modificaciones.

Los niosomas se formularon utilizando Gelot 64 (G64) como tensioactivo y dos ácidos grasos no saturados, oleico (OA) y linoleico (LA), los cuales se usan de manera habitual en formulaciones farmacológicas como potenciadores de la penetración en la piel de principios activos. Los niosomas se caracterizaron determinando los siguientes parámetros: tamaño medio y distribución de tamaños, estabilidad y eficacia de encapsulación del RSV.

Artículo 4. D. Pando, M. Matos, G. Gutiérrez, C. Pazos. *Formulation of resveratrol entrapped niosomes for topical use*

Colloids and Surfaces B: Biointerfaces. En proceso de evaluación.

Contribución personal al trabajo

Gracias a la experiencia adquirida durante mi estancia en la Universidad de Cagliari, llevé a cabo los experimentos de absorción percutánea de RSV en piel de cerdo recién nacido utilizando celdas Franz, con la ayuda de Andrea González, técnico de laboratorio. Asimismo, realicé el diseño de experimentos y el análisis de la varianza (ANOVA). Finalmente, preparé el manuscrito inicial de la publicación con la colaboración de la Dra. Gemma Gutiérrez y la supervisión de mis directoras de Tesis, Dra. Maria Matos y Prof. Carmen Pazos.

Elsevier Editorial System(tm) for Colloids and Surfaces B: Biointerfaces
Manuscript Draft

Manuscript Number:

Title: Formulation of resveratrol entrapped niosomes for topical use

Article Type: Full Length Article

Keywords: niosomes, resveratrol, thin film hydration method, ethanol injection method, penetration enhancer, topical use

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Manuscript Region of Origin: SPAIN

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Niosomes were prepared by two different methods: a thin film hydration method with minor modifications followed by a sonication stage (TFH-S), and an ethanol injection modified method (EIM).
Niosomes prepared with the EIM method were in the range of 299-402 nm, while the TFH-S method produced larger niosomes in the range of 293-496 nm. Moreover, niosomes with higher RSV entrapment efficiency (EE) and better stability were generated by the EIM method.
Ex-vivo transdermal experiments, carried out in Franz diffusion cells on newborn pig skin, indicated that niosomes prepared by the EIM method were more effective for RSV penetration in epidermis and dermis (EDD), with values up to 21% for both penetration enhancers tested.
The EIM method, yielded the best RSV-entrapped niosomes, seems to be the more suitable for scaling up.

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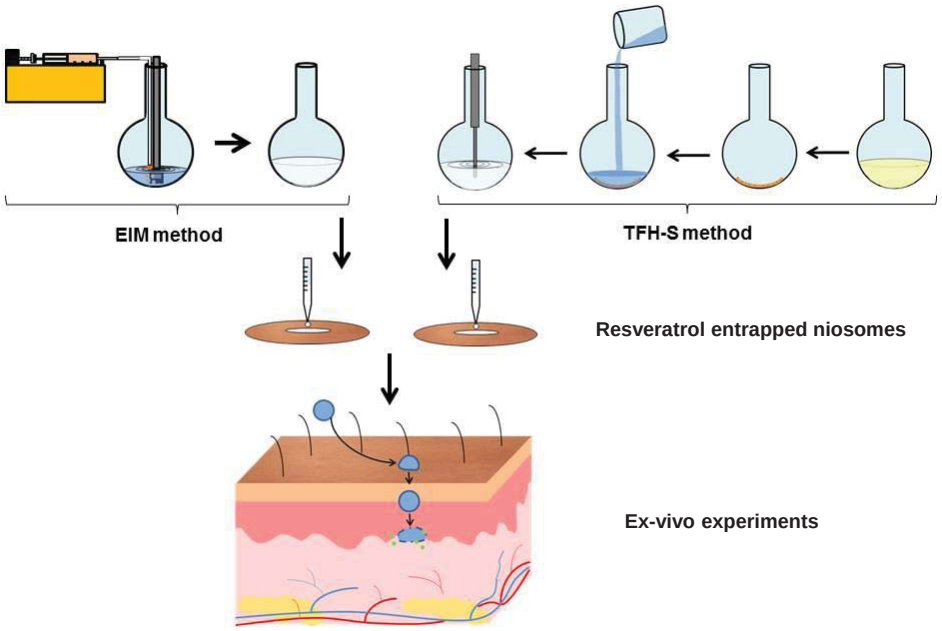

Highlights

- Resveratrol entrapped niosomes for topical use were formulated
- Thin film hydration and ethanol injection (EIM) were used as preparation methods
- Oleic and linoleic acids were compared as skin penetration enhancers
- EIM produced small size niosomes with high encapsulation efficiency
- Smaller niosomes were more effective for resveratrol skin penetration

Formulation of resveratrol entrapped niosomes for topical use

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Abstract

A new approach to the formulation of resveratrol (RSV) entrapped niosomes for topical use is proposed in this work.

Niosomes were formulated with Gelot 64 (G64) as surfactant, and two skin-compatible unsaturated fatty acids (oleic and linoleic acids), commonly used in pharmaceutical formulations, as penetration enhancers.

Niosomes were prepared by two different methods: a thin film hydration method with minor modifications followed by a sonication stage (TFH-S), and an ethanol injection modified method (EIM). Niosomes prepared with the EIM method were in the range of 299-402 nm, while the TFH-S method produced larger niosomes in the range of 293-496 nm. Moreover, niosomes with higher RSV entrapment efficiency (EE) and better stability were generated by the EIM method.

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The EIM method, yielded the best RSV-entrapped niosomes, seems to be the more suitable for scaling up.

Keywords: niosomes, resveratrol, thin film hydration method, ethanol injection method, penetration enhancer, topical use.

1. Introduction

Resveratrol (RSV) is a natural polyphenol found in a wide variety of plants that has both chemopreventive and therapeutic effects, because of its anti-oxidant, anti-inflammatory, cardioprotective, and anti-tumour properties. However, its applications are restricted because it is easily oxidizable, has low solubility in water, short biological half-life, and rapid metabolism and elimination (Caddeo, *et al*. 2013; Pando, *et al*. 2013a; Pando, *et al.* 2013b; Scognamiglio, *et al.* 2013; Matos, *et al.* 2014). Moreover, it is an extremely photosensitive molecule, and exposure to light leads to an irreversible change from the active *trans* isomer to the inactive *cis* isomer. Thus, *trans*-resveratrol should be encapsulated before being administered either for food or topical applications.

Because of its chemopreventive and antioxidant properties, RSV is considered to be an interesting drug for incorporation into dermatological preparations. Special attention has been paid to its topical application in different physiological and pathological conditions, such as skin cancer prevention or psoriasis treatment (Jang, *et al*. 1997; Caddeo, *et al*. 2013; Scognamiglio, *et al*. 2013). The drug delivery into the skin has the advantage that high drug concentrations are located at specific sites of action. For this reason, the *ex-vivo* percutaneous absorption of RSV in different nanocarriers has been widely investigated in the last years (Sinico and Fadda 2009, Pando, *et al*. 2013b, Scognamiglio, *et al*. 2013, Marianecci, *et al*. 2014).

The intercellular lipids of the human stratum corneum consist mainly of cholesterol, ceramides and free fatty acids, structurally organized into multilamellar bilayers, which dictate the overall skin permeability properties.

Liposomal delivery systems have been used as a promising approach to overcome the limited permeability of drug across the stratum corneum of skin (Srisuk, *et al*. 2012). However, over the last two decades, niosomes are preferred over liposomes because of their higher chemical stability, lower cost, and the lower efficiency of liposomes for drug delivery across the skin (Sinico and Fadda 2009, Marianecci, *et al*. 2012). Niosomes are vesicles formed by the auto-assembling of non-ionic surfactants in aqueous media resulting in closed bilayer structures (Uchegbu, and Vyas, 1998). Non-ionic surfactants can improve the solubility of some poorly soluble drugs enhancing transdermal delivery by encapsulation. These vesicular systems also provide sustained drug release to prolong its action (Kumar and Rajeshwarrao, 2011).

The purpose of this work is to propose a new approach to formulate RSV-entrapped niosomes for topical delivery by comparing two specific methods of preparation: a thin film hydration method with minor modifications, followed by a sonication stage (TFH-S), and an ethanol injection modified method (EIM).

Niosomes were formulated with Gelot 64 (G64) as surfactant, and two skin-compatible unsaturated fatty acids as penetration enhancers, commonly used in pharmaceutical

formulations: oleic acid (OA) and linoleic acid (LA) (Rita and Lakshmi, 2012). Niosomes were characterized in terms of size, morphology, and stability. *Ex-vivo* transdermal experiments, carried out in Franz diffusion cells on newborn pig skin, enabled to study the influence of niosomes formulation and preparation method on RSV skin delivery.

2. Materials and methods

2.1. Materials

RSV, OA and LA, all of them with purity >99%, were supplied by Sigma-Aldrich (Germany). G64 was a gift from Gattefossé (France). Methanol, acetonitrile, 2-propanol, and acetic acid of HPLC-grade were purchased from Sigma-Aldrich (USA). Deionized water was used in all experiments.

2.2. Niosomes preparation

Niosomes containing RSV were prepared by the following methods:

2.2.1. Thin film hydration-sonication method (TFH-S)

The TFH method (Bangham, *et al.* 1965, Baillie, *et al.* 1985) was utilized with minor modifications, followed by a sonication stage (TFH-S).

Accurately weighed amounts of G64 and penetration enhancer (OA or LA) in different weight ratios, in the range of 1:0.5 to 1:1.5, were dissolved in 6.25 mL of an absolute ethanol solution containing a known concentration of RSV, and placed in a 100 mL round bottom flask. Then, ethanol was removed at 40 ºC under reduced pressure in a rotary evaporator (Buchi, Switzerland). The dried film was hydrated with 12.5 mL of deionized water at 60 ºC to achieve a RSV concentration of 1 mg/mL. The resulting solution was further sonicated for 30 minutes (CY-500 sonicator, Optic Ivymen System, Spain), using 45% amplitude, 500 W power, and 20 kHz frequency.

2.2.2. Ethanol injection modified method (EIM)

The conventional ethanol injection method, first described in 1973 (Batzri and Korn, 1973), offers advantages such as simplicity, absence of potentially harmful chemicals, and suitability for scaling-up (Wagner, *et al*. 2002; Pham, *et al.* 2012).

Appropriate weighed amounts of G64 and stabilizer (OA or LA) in different weight ratios, from 1:0.5 to 1:1.5, were dissolved in 6.25 mL of an absolute ethanol solution containing a known concentration of RSV. Then, this solution was injected, with a syringe pump (KDScientific, USA) at a flow of 120 mL/h, into deionized water at 60ºC, stirring at 15000 rpm with a homogenizer (SilentCrusher M, rotor model 22G, Heidolph, Germany). Although spontaneous niosomes formation occurs as soon as the organic solution is in contact with the aqueous phase (Pham, *et al*. 2012), vigorous agitation is

needed to obtain narrower size distributions. Once niosomes were formed, ethanol was removed at 40 ºC under reduced pressure in a rotary evaporator.

2.3. Niosomes size

Mean (Z-Average) size and polydispersity index (PDI) of niosomes were determined via Dynamic Light Scattering (DSL) using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). Three independent samples were taken from each formulation, and measurements were carried out three times at room temperature without dilution.

2.4. Niosomes morphology

Morphological analysis of niosomes was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM (Japan). A drop of the niosomal formulation was placed on a carbon-coated copper grid, and the sample excess was removed with filter paper. Then a drop of 2% (w/v) PTA (phosphotungstic acid solution) was applied to the carbon grid and left to stand for 2 minutes. Once the excess staining agent was removed with filter paper, the sample was air-dried and the thin film of stained niosomes was observed with the transmission electron microscope.

2.5. Niosomes stability

The stability of niosomes was determined by measuring backscattering (BS) profiles in a Turbiscan Lab® Expert apparatus (Formulaction, France) provided with an Ageing Station (Formulaction, France). Undiluted niosomes samples were placed in the cylindrical glass test cells and backscattered light was monitored as a function of time and cell height for 15 days, every 3 hours, at 30 °C. The optical reading head scans the sample in the cell, providing BS data every 40 μm in % relative to standards as a function of the sample height (in mm). These profiles build up a macroscopic fingerprint of the niosomes at a given time, providing useful information about changes in niosomes size distribution or appearance of a creaming layer or a clarification front with time (Pando, *et al*. 2013a).

2.6. Niosomes entrapment efficiency (EE)

Entrapped RSV was removed from free RSV by dialysis. A 2 mL sample was placed into a dialysis bag, immersed in 1000 mL of deionized water at room temperature, and stirred at 500 rpm for 2 hours. Dialyzed and non-dialyzed samples were diluted 1:10 (v/v) with methanol to facilitate the rupture of vesicle membrane and to extract RSV from vesicles. Then, RSV was analysed by chromatography (RP-HPLC) (HP series 1100 chromatograph, Hewlett Packard, USA). The system was equipped with a UV/VIS absorbance detector HP G1315A and a fluorescence detector 1260 Infinity A (Agilent Technologies, USA). A 305 nm wavelength was used for UV/VIS detector while fluorescence detector used 310/410 nm of $\lambda_{excitation}/\lambda_{emission}$ at 310/410 nm. The column

144 was a Zorbax Eclipse Plus C_{18} of 5 µm particle size, 4.6 mm × 150 mm (Agilent
145 Technologies, USA).

146 The mobile phase consisted of a mixture of (A) 100% milliQ-water and (B) 100%
147 methanol with gradient elution at 0.8 mL/min. The step gradient started with a mobile
148 phase of 80% (A) running 100% mobile phase (B) in minute 5 for 10 minutes. The
149 mobile phase (B) was fed for 2 minutes after each injection to prepare the column for
150 the next sample. The separation was carried out at 30°C.

151 *2.7. Ex-vivo skin penetration and permeation studies*

152 Experiments were carried out in vertical Franz cells with an effective diffusion area of
153 0.785 cm^2, and using the skin of newborn pig. The skin, previously frozen at -80°C,
154 was pre-equilibrated in saline solution at 25°C for 1 hour. Then, the skin was placed
155 onto the Franz cell and sandwiched with the stratum corneum (SC) side facing the
156 donor compartment. The receptor container, thermostated at 37 ± 1°C, was filled with
157 5.5 mL saline solution (0.9% w/v NaCl), and was continuously agitated with magnetic
158 stirring.

159 A 100 µL sample was applied onto the skin, in the donor compartment (*n* = 3 per
160 formulation) during 8 hours. After this period of time and once the skin was removed
161 from the Franz cell, it was gently rinsed with deionized water and dried.

162 To determine the amount of RSV that did not penetrate into the deeper layer of the skin
163 (*i.e.* epidermis and dermis (EDD) passing through the SC), a separation of SC from
164 skin was carried out. This was carried out by stripping the SC layer with adhesive tape
165 (Tesa AG, Germany). The RSV present in SC and EDD was extracted with methanol.
166 This method had been previously validated by histological examination of stripped skin
167 (Manconi, *et al*. 2005).

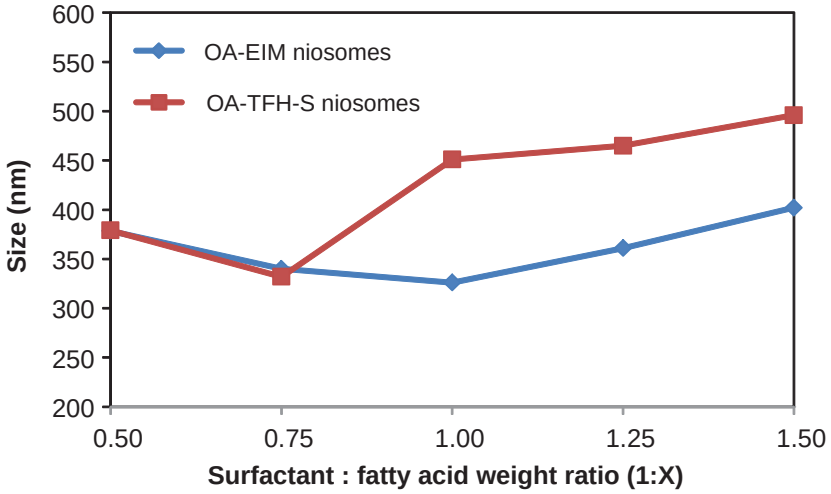
168 Receiver compartment samples were lyophilized and then RSV was extracted with
169 methanol. RSV content in SC, EDD and saline reception solution was determined by
170 RP-HPLC.

171 *2.8. Statistical analysis*

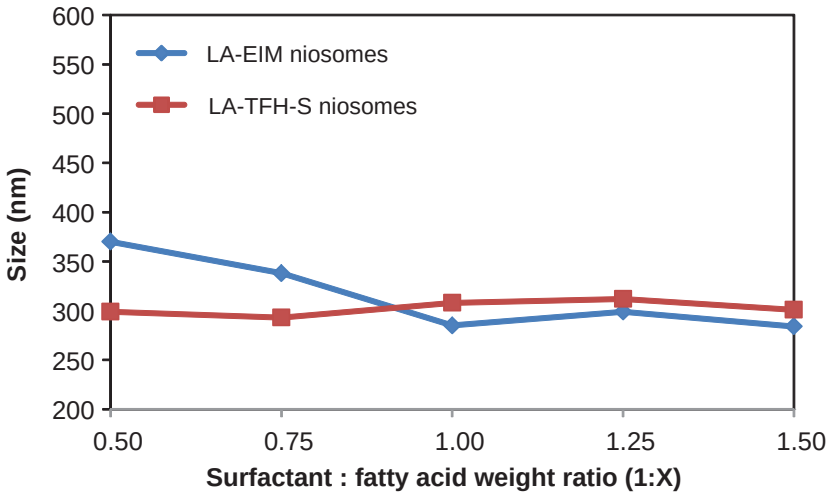
172 All data were expressed as the mean ± SD (standard deviation) of three independent
173 experiments, and statistical analysis of the data was carried out (ANOVA). Fisher's test
174 ($p<0.05$) was used to calculate the least significance difference (LSD) using statistical
175 software (Microsoft Excel 2010).
176
177 **3. Results and discussion**

178 *3.1. Mean size and size distribution of niosomes*

The mean sizes of niosomes formulated with G64 as surfactant, and OA or LA as penetration enhancer, at different weight ratios are shown in Figure 1.



(a)



(b)

Figure 1. Effect of surfactant (G64) to fatty acid weight ratio on niosomes size prepared by EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

6

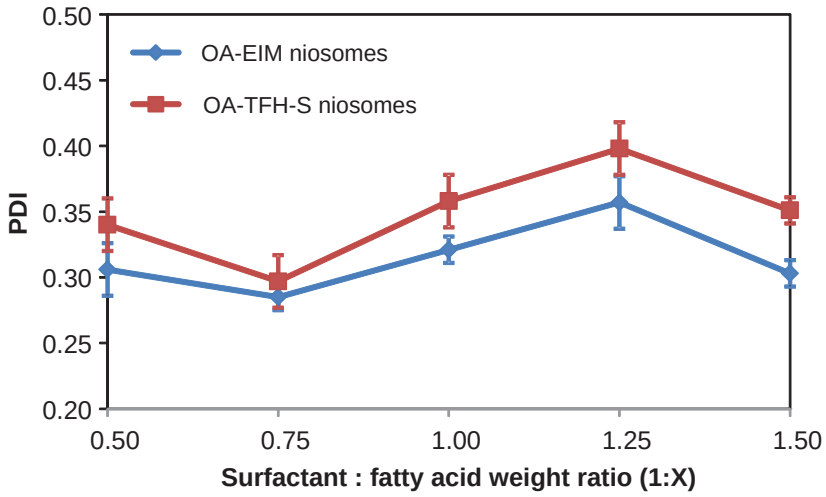
Niosomes prepared with LA as penetration enhancer showed smaller sizes than those prepared using OA ($p < 0.05$). Variations in niosome size up to 40% with TFH-S method, and up to 30% for EIM method were observed, depending on the type and concentration of fatty acid.

Figure 1(a) shows the niosome size as a function of the G64 : OA weight ratio for both preparation methods. It was observed that niosomes prepared by the EIM method had smaller size, in the range 326-402 nm. Niosomes prepared with the TFH-S method showed a higher variation with this parameter ($p < 0.05$). Moreover, while similar sizes were obtained with both methods for G64 : OA weight ratios of 1:0.5 and 1:0.75, big discrepancies were observed for larger weight ratios, and larger niosomes were obtained by the TFH-S method.

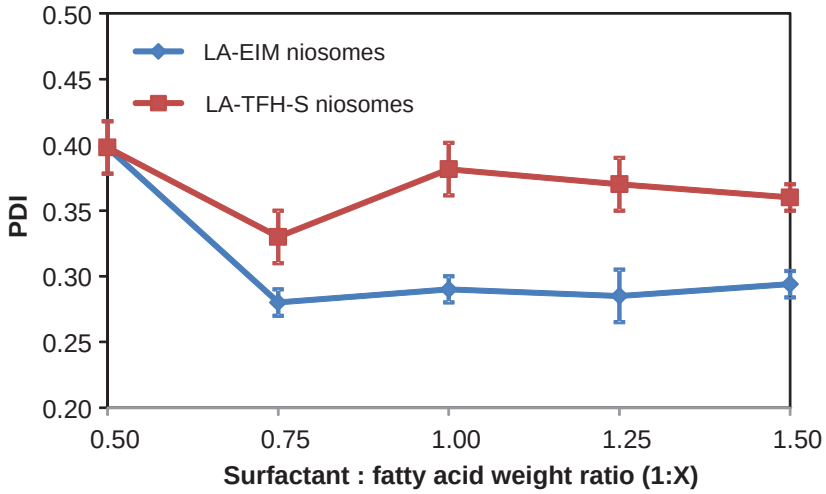
As shown in Figure 1(b) an opposite trend was observed when niosome size was plotted versus the G64 : LA weight ratio for both preparation methods. Niosomes prepared by the EIM method exhibited large variations ($p < 0.05$), while no significant differences were found with the TFH-S method for all the G64 : LA weight ratios used.

These results show that the selection of LA or OA as penetration enhancer involves significant differences in niosomes size, and are highly dependent on both the surfactant : fatty acid weight ratio and the preparation method.

PDI values of niosomes formulated with G64 as surfactant, and OA or LA as penetration enhancer, at different weight ratios are shown in Figure 2.



(a)



(b)

Figure 2. Effect of surfactant (G64) to fatty acid weight ratio on PDI of niosomes prepared using EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

For niosomes made of G64 and OA as penetration enhancer, Figure 2(a), there was significant relation between G64 : OA weight ratio and sample PDI ($p < 0.05$) for both preparation methods. The same trend was observed regarding the best PDI values at a

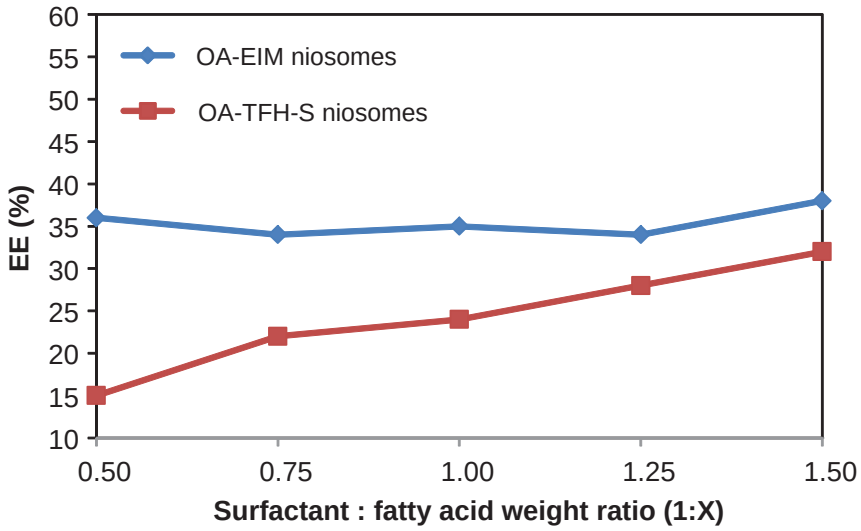
weight ratio G64 : OA of 1:0.75 for both methods. For the G64 : OA weight ratio range studied, the EIM method yielded lower PDI values.

There was also a significant relation between G64 : LA weight ratio and PDI of the sample ($p < 0.05$) for niosomes made with G64 and LA as penetration enhancer, Figure 2(b). The best PDI values corresponded to the same weight ratio (1:0.75) for both methods. G64–LA niosomes prepared by the EIM method showed lower PDI values than G64–LA niosomes prepared by the TFH-S method.

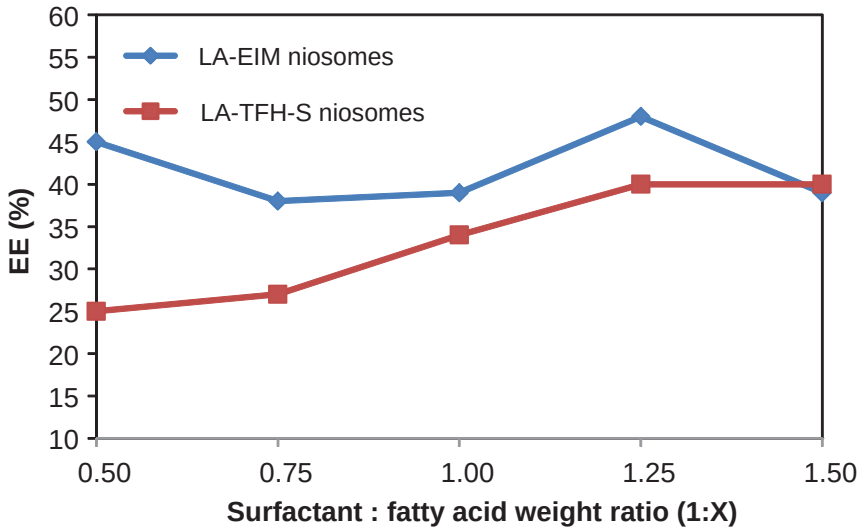
These results prove that the G64 : fatty acid weight ratio is significantly relevant in order to reach low PDI value, which involves a monodisperse distribution. The best PDI value is obtained for weight ratio 1: 0.75 independently of the niosomes formulation and method of preparation.

3.2. Niosomes entrapment efficiency (EE)

Figure 3 shows the EE values obtained as a function of G64 : fatty acid weight ratio.



(a)



(b)

Figure 3. Effect of surfactant (G64) to fatty acid weight ratio on EE of niosomes prepared using EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

EE of RSV showed a clear trend with the amount of OA used as penetration enhancer, Figure 3(a), for niosomes prepared with the TFH-S method ($p < 0.05$), the higher G64 : OA weight ratio, the higher EE. However, slight variations were obtained on EE for

G64-OA niosomes prepared with the EIM method, which were in all cases higher than those obtained by TFH-S method.

For G64-LA niosomes, there was a relationship between EE and amount of LA used, slightly more evident for niosomes prepared by TFH-S method ($p < 0.05$), since EE increased with G64 : LA weight ratio. Also in this case, niosomes prepared by EIM method showed higher EE than niosomes prepared by TFH-S method, except for G64 : LA weight relation of 1:1.5, where similar EE values were obtained with both methods.

These results show a clear dependence of EE on the preparation method ($p < 0.05$), higher values being obtained for niosomes prepared with the EIM method. Only the niosomes formulated at G64 : LA weight ratio of 1:1.5 have similar EE values for both methods.

It had been previously reported that the presence of the active compound did not change the average size of vesicles (Manca, *et al*. 2013). Moreover, some authors found relation between vesicle size and EE, being the largest vesicles the ones with the highest EE (Maestrelli, *et al*. 2006; Srisuk, *et al*. 2012; Cadena, *et al*. 2013). However, in the present work it was not observed a clear relationship between EE and niosomes mean size.

3.3. Ex-vivo skin penetration and permeation studies

The release of RSV entrapped niosomes across a series of barriers and anatomical structures of the skin, as function of different formulations and preparation methods, was studied. Skin penetration occurs by diffusion of the active compound across the skin layers into the receptor phase, *i.e.* subcutaneous fluids and blood vessels

Release experiments were carried out using skin of newborn pig, as it is known that pig skin is a good substitute in ex-vivo permeation experiments due to its similarity with the SC of human skin in terms of lipid composition. Although it presents a marked difference in thickness, newborn pig SC is considerably thinner than that of adult pigs, and more similar to the human skin, even if the number of hair follicles is higher (Pando, *et al*. 2013b). Several studies have been carried out with newborn pig skin, confirming its suitability for skin permeation screenings (Manconi, *et al*. 2011).

RSV effects on the skin only appear when RSV penetrates to the deeper layers of skin (EDD), being necessary to cross the SC. Due to the difficulty to precisely separate epidermis and dermis, in this study three parts were distinguished: SC, EDD and receptor fluid (RC).

The amount of RSV accumulated into these parts (SC, EDD and RC) was analysed by RP-HPLC. These measurements enabled to make a mass balance of RSV in the system to reinforce the method applied, since total RSV lost was less than 10% in all cases.

RSV penetration into the deeper layers of skin (EDD), using different formulations and niosomes preparation methods, are shown in Figure 4.

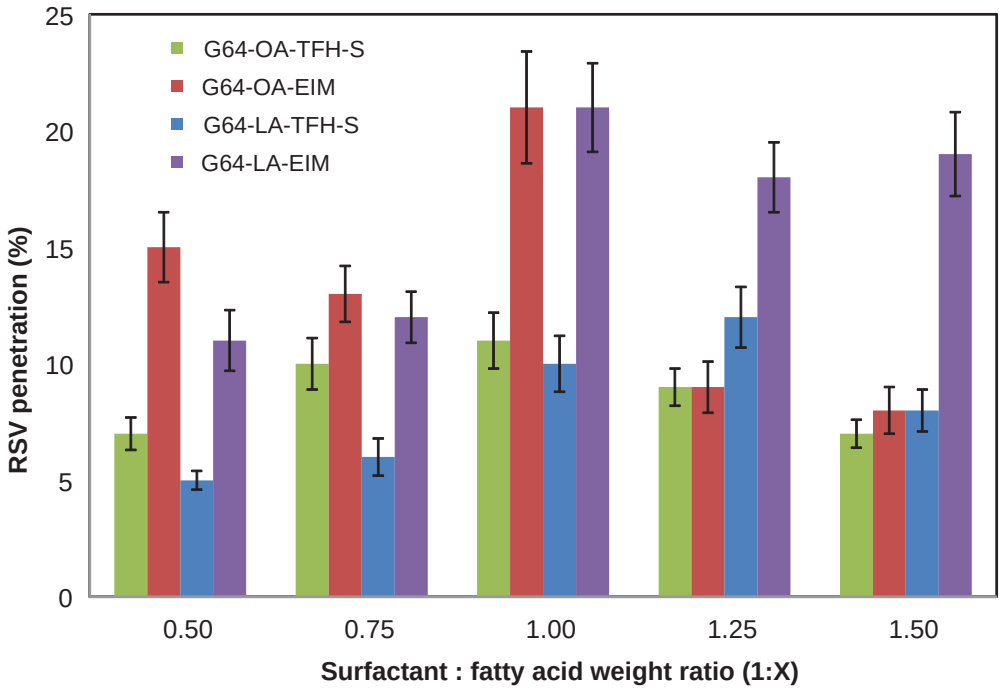


Figure 4. RSV penetration in epidermis and dermis (EDD): influence of formulation and niosomes preparation method

A close correlation between the amount of fatty acid used and RSV penetration ($p < 0.05$) was observed, being stronger for niosomes prepared with the EIM method, for both penetration enhancers, OA and LA.

It was also clear the dependence between RSV penetration in EDD and the niosomes preparation method ($p < 0.05$), being the EIM more effective for all formulations tested ($p< 0.05$). Niosomes prepared with this method at a weight ratio of 1:1 for both penetration enhancers were the most effective, showing RSV penetration values up to 21%.

In order to compare RSV penetration with niosomes size, PDI and EE values, all these data are summarized in Table 1.

289 Table 1. Mean size (Z-average), PDI, EE, and RSV penetration into the EDD layer for niosomes
290 formulated with G64 as surfactant and OA or LA as penetration enhancer, using TFH-S
291 or EIM as preparation methods

Preparation method	Fatty acid (FA)	G64 : FA weight ratio	Mean size (nm)	PDI	EE (%)	RSV penetration (%)
TFH-S	OA	1:0.5	379 ± 36	0.34 ± 0.01	15 ± 1	7.0 ± 0.7
		1:0.75	332 ± 25	0.30 ± 0.01	22 ± 2	10.0 ± 1.1
		1:1	451 ± 42	0.36 ± 0.02	24 ± 2	11.0 ± 1.2
		1:1.25	465 ± 32	0.40 ± 0.02	28 ± 2	9.0 ± 0.8
		1:1.5	496 ± 39	0.35 ± 0.02	32 ± 2	7.0 ± 0.6
	LA	1:0.5	299 ± 19	0.40 ± 0.02	25 ± 2	5.0 ± 0.4
		1:0.75	293 ± 21	0.33 ± 0.02	27 ± 2	6.0 ± 0.8
		1:1	308 ± 28	0.38 ± 0.02	34 ± 2	10.0 ± 1.2
		1:1.25	312 ± 30	0.37 ± 0.02	40 ± 3	12.0 ± 1.3
		1:1.5	301 ± 30	0.36 ± 0.01	40 ± 3	8.0 ± 0.9
EIM	OA	1:0.5	378 ± 36	0.31 ± 0.01	36 ± 2	15.0 ± 1.5
		1:0.75	340 ± 33	0.29 ± 0.01	34 ± 3	13.0 ± 1.2
		1:1	326 ± 33	0.32 ± 0.01	35 ± 2	21.0 ± 2.4
		1:1.25	361 ± 30	0.36 ± 0.02	34 ± 2	9.0 ± 1.1
		1:1.5	402 ± 36	0.30 ± 0.02	38 ± 2	8.0 ± 1.0
	LA	1:0.5	370 ± 26	0.40 ± 0.02	45 ± 3	11.0 ± 1.3
		1:0.75	338 ± 25	0.28 ± 0.01	38 ± 3	12.0 ± 1.1
		1:1	285 ± 26	0.29 ± 0.01	39 ± 3	21.0 ± 1.9
		1:1.25	299 ± 28	0.28 ± 0.02	48 ± 3	18.0 ± 1.5
		1:1.5	284 ± 28	0.29 ± 0.01	39 ± 2	19.0 ± 1.8

292 As a general trend, higher RSV penetration corresponded to niosomes with smaller
293 mean sizes. A similar behaviour had been reported by other authors who attributed this
294 effect to the size of the small vesicles that led to an increase vesicle/skin interface
295 enhancing interactions with skin lipids, and increasing transdermal fluxes (Verma, *et al*.
296 2003; Maestrelli, *et al*. 2006; Srisuk, *et al*. 2012; Manca, *et al.* 2013).

297 However, it was observed that formulations with the best EE were not the most suitable
298 regarding RSV penetration, as it had been previously reported by other authors
299 (Maestrelli, *et al*. 2006; Srisuk, *et al*. 2012; Kong, *et al*. 2013).

300 *3.4. Characterization of the optimum niosomes*

301 Stability with time was examined for the best formulations to compare the effect of both
302 OA and LA as penetration enhancers, as well as niosomes preparation method. Hence,
303 a G64 : fatty acid weight ratio of 1:1 was selected, since this ratio showed successful
304 results with respect to EE and RSV transdermal delivery. Morphology of these
305 niosomes was also confirmed by TEM.

306 Figure 5 shows four negative stain micrographs of RSV entrapped niosomes obtained
307 with the best aforementioned formulations.

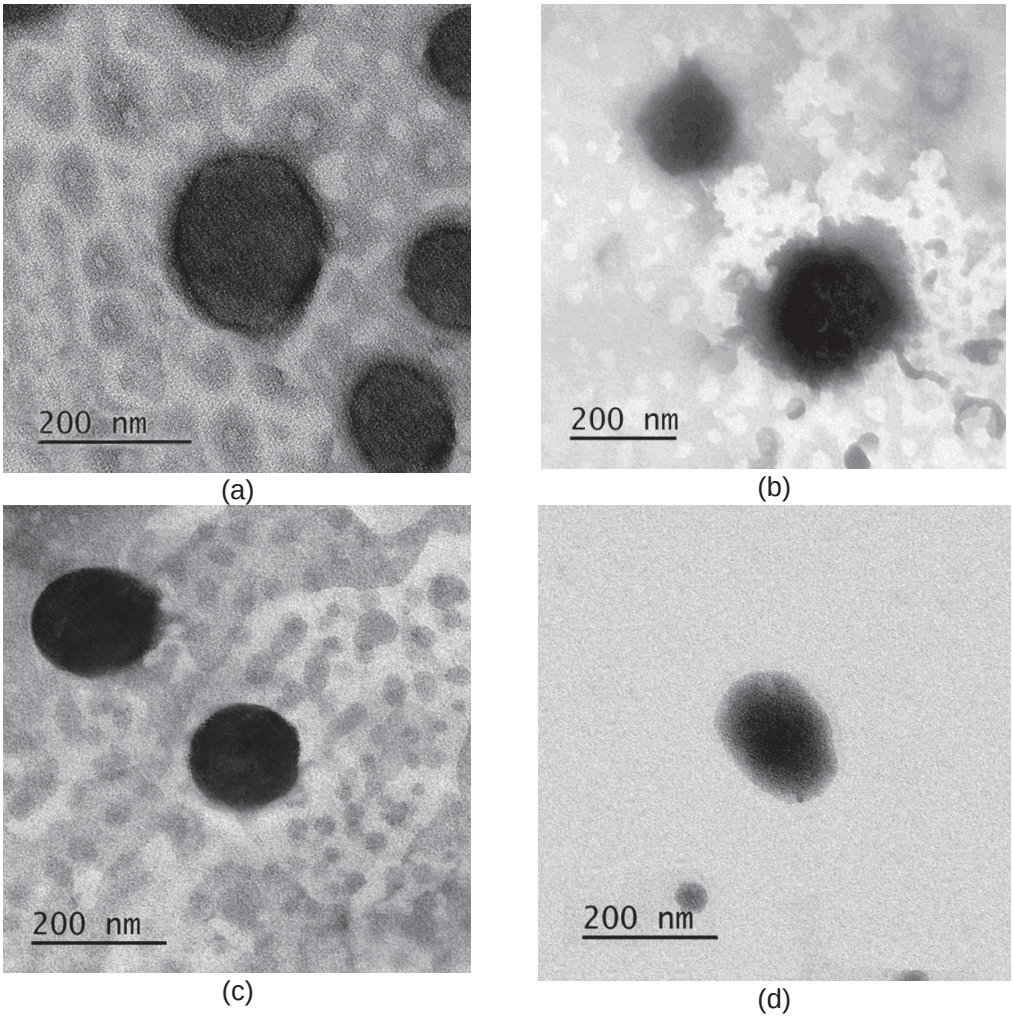


Figure 5. TEM micrographs of different niosomes formulated with a surfactant : fatty acid weight ratio of 1:1. (a) G64-OA-TFH-S; (b) G64-OA–EIM; (c) G64- LA-TFH-S; (d) G64-LA-EIM

Dark-stained niosomes were obtained as a result of the strong interactions between surfactant and phosphotungstic acid, allowing a selective electrons deposit in the sample, which enhanced structural details. Micrographs showed circular and dark structures corresponding to spherical niosomes of approximately 300-400 nm, according to DLS measurements. It can be clearly observed that mean size of niosomes prepared by EIM method, Figures 5(b) and 5(d), were smaller than those prepared by TFH-S method, Figures 5(a) and 5(c). Niosomes formulated with OA, Figures 5(a) and 5(b) also presented larger values.

Figure 6 shows the BS profiles of different niosomal samples examined for 15 days every 3 hours.

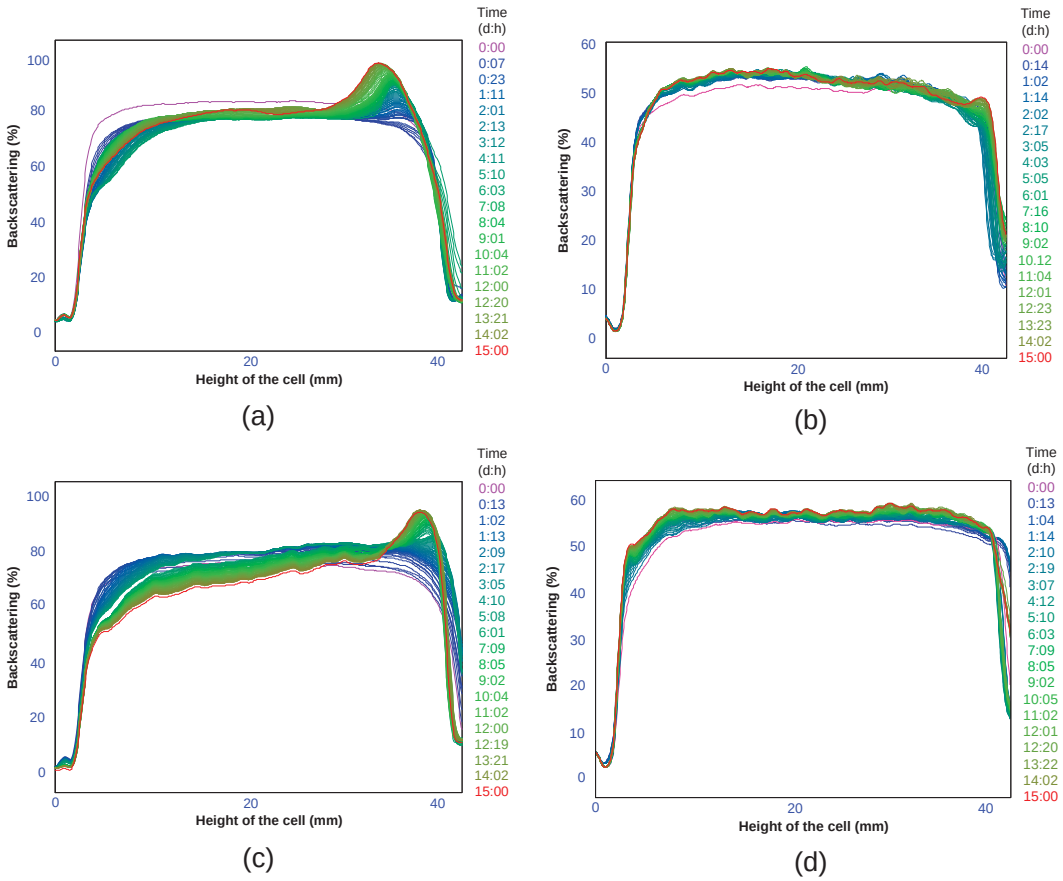


Figure 6. BS profiles of different niosomes formulated with a surfactant : fatty acid weight ratio of 1:1. (a) G64-OA-TFH-S; (b) G64-OA –EIM; (c)G64- LA-TFH-S; (d) G64-LA-EIM

Figures 6(b) and 6(d) indicate the higher stability of niosomes prepared by EIM method, using either OA or LA. In both cases, BS variation (ΔBS) was lower than 10% being homogenous along the cell, which means that there were no significant changes in niosomes size, remaining the sample stable with no destabilization phenomena, such as aggregation or coalescence. Moreover, not significant creaming or sedimentation phenomena were observed in these samples during the monitoring time.

However, niosomes prepared using TFH-S method, Figures 6(a) and 6(c), showed creaming phenomena evidenced by an increase of BS at the top of the cell, and a simultaneous decrease at the bottom, with ΔBS up to 20%. For niosomes formulated with OA this effect could be attributed to their larger sizes, and hence migration phenomena could easily take place due to differences between densities of niosomes and aqueous external phase. Similar behaviour has been found in other colloidal systems (Gutiérrez, *et al*. 2014).

4. Conclusions

The present work indicates that the EIM method produces niosomes with smaller mean sizes, narrower size distributions, higher EE and stability than those prepared with the TFH-S method. Furthermore, the EIM method is more suitable for scaling up.

A clear dependence was observed between RSV penetration in EDD and niosomes preparation method, being the EIM method more effective for all formulations tested, which can be related to the smaller niosomes mean size obtained with this method.

An optimum dosage of penetration enhancer is needed to obtain high RSV penetration values, although no significant differences were observed between both, OA and LA, enhancers tested.

No relationship between EE and niosomes mean size, as well as with RSV penetration was found. Further research is needed to have a better understanding of the mechanisms involved in RSV skin delivery through niosomes.

Acknowledgements

This work was supported by the Ministerio de Ciencia e Innovación (MICINN, Spain), under the Grant MICINN-10-CTQ2010-20009-C02-01, and co-financed by the Consejería de Educación y Ciencia del Principado de Asturias (Ref. FC-04-COF-50-MEC and PCTI Asturias 2006-2009, Ref. EQP06-024).

D. Pando acknowledges receipt of a graduate fellowship from Severo Ochoa Program (Principado de Asturias, Spain).

The authors thank Prof. José Coca (University of Oviedo) for fruitful discussions, and A. González for her kind help with lab work.

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423

4.GENERAL CONCLUSIONS

From the results presented in this Thesis and in the attached publications the following conclusions could be reached:

1. Niosomes prepared from Span 80-cholesterol or Span 60-cholesterol are able to encapsulate resveratrol (RSV). However, a proper selection of niosomes formulation and preparation method is required depending on the final product characteristics. High speed mechanical agitation produces niosomes with smaller average size and narrower size distribution, but the entrapment efficiency (EE) is low. The use of sonication as a second preparation stage decreases slightly niosomes average size and enables to obtain a size distribution considerably narrower.

2. Niosomes formulated with Plurol Oleique and Peceol are able to entrap high RSV amounts (~82%). Although physicochemical properties of liposomes are similar to niosomes, the latter are more suitable as carriers for transdermal RSV delivery, as they increase the local effect of the active ingredient.

3. Niosomes formulated with Span 60 or Maisine 35-1 as surfactants and dodecanol as membrane stabilizer are appropriate for RSV entrapment. Furthermore, they can be used as yogurt additives, since they do not affect the textural properties of this dairy product.

4. Gelot 64, as surfactant, and oleic acid or linoleic acid, as skin penetration enhancer, are suitable formulations to produce RSV entrapped niosomes for topical use. Ethanol injection modified (EIM) method is the most effective, as it allows to obtain the most stable niosomes, which show the smallest mean size, the narrowest size distributions, and the highest EE and RSV skin penetration.

5.CONCLUSIONES GENERALES

A partir de los resultados presentados en esta Tesis y en las publicaciones adjuntas, se pueden obtener las siguientes conclusiones:

1. Aunque los niosomas preparados a partir de Span 80-colesterol o Span 60-colesterol resultan válidos para la encapsulación de resveratrol (RSV), se requiere una adecuada selección de la formulación y del método de preparación de estas vesículas teniendo en cuenta las características del producto final. Los niosomas preparados con velocidades de agitación elevadas presentan un tamaño medio pequeño y una distribución de tamaños estrecha, pero su eficacia de encapsulación es baja. La aplicación de sonicación, como segundo paso en el método de preparación, reduce ligeramente el tamaño medio de los niosomas y permite llegar a una distribución de tamaños mucho más estrecha.

2. Los niosomas formulados con Plurol Oleique y Peceol permiten la encapsulación de elevadas cantidades de RSV (~82%). Aunque las propiedades fisicoquímicas de los liposomas son similares a las de los niosomas, estos últimos resultan más efectivos para la liberación transdérmica del RSV, lo que incrementa la eficacia del principio activo a nivel local.

3. Los niosomas formulados con Span 60 o Maisine 35-1 como tensioactivos y dodecanol como estabilizante de membrana resultan apropiados para la encapsulación de RSV. Además, se pueden usar como aditivos de yogur, ya que su presencia no altera las propiedades texturales de este producto lácteo.

4. El Gelot 64 como tensioactivo y el ácido oleico o linoleico como potenciador para favorecer la penetración en la piel constituyen una formulación adecuada para la encapsulación de RSV con fines tópicos. El método de inyección de etanol modificado (EIM) es el que resulta más eficaz, ya que produce los niosomas más estables, con tamaños medios más pequeños, distribuciones de tamaño más estrechas, así como más elevadas eficacias de encapsulación y de penetración de RSV en la piel.