



ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ
ΤΜΗΜΑ ΒΙΟΧΗΜΕΙΑΣ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ



ΕΘΝΙΚΟ ΙΔΡΥΜΑ ΕΡΕΥΝΩΝ
ΙΝΣΤΙΤΟΥΤΟ ΧΗΜΙΚΗΣ ΒΙΟΛΟΓΙΑΣ

ΔΙΙΔΡΥΜΑΤΙΚΟ ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ ΒΙΟΕΠΙΧΕΙΡΕΙΝ



ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

ΣΧΗΜΑΤΙΣΜΟΣ ΚΑΙ ΠΡΟΕΤΟΙΜΑΣΙΑ ΠΑΡΑΣΚΕΥΗΣ ΝΙΟΣΩΜΑΤΩΝ ΚΑΙ
ΛΙΠΟΣΩΜΑΤΩΝ ΠΟΥ ΠΕΡΙΕΧΟΥΝ ΒΙΟΔΡΑΣΤΙΚΕΣ ΕΝΩΣΕΙΣ

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ: ΛΕΩΝΙΔΑΣ ΔΗΜΗΤΡΙΟΣ, ΚΑΘΗΓΗΤΗΣ ΒΙΟΧΗΜΕΙΑΣ, ΤΜΗΜΑ
ΒΙΟΧΗΜΕΙΑΣ & ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ, ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΛΙΒΑΝΙΟΣ ΔΗΜΗΤΡΙΟΣ
Α.Μ. 00040
ΑΘΗΝΑ, 2019



UNIVERSITY OF THESSALY
SCHOOL OF HEALTH SCIENCES
DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY



NATIONAL HELLENIC RESEARCH FOUNDATION
INSTITUTE OF CHEMICAL BIOLOGY

**INTERSTITUTIONAL PROGRAM OF POSTGRADUATE STUDIES
IN
BIOENTREPRENEURSHIP**



MASTER THESIS

**FORMULATION AND PREPARATION OF NIOSOMES AND LIPOSOMES
CONTAINING BIOACTIVE COMPOUNDS**

**SUPERVISOR: LEONIDAS DEMETRES D., PROFESSOR BIOCHEMISTRY, DEPARTMENT OF
BIOCHEMISTRY AND BIOTECHNOLOGY, UNIVERSITY OF THESSALY**

**LIVANIOS DIMITRIOS
R.N. 00040
ATHENS, 2019**

Η παρούσα διπλωματική εργασία εκπονήθηκε στο πλαίσιο σπουδών
για την απόκτηση του Μεταπτυχιακού Διπλώματος Ειδίκευσης στο

ΒΙΟΕΠΙΧΕΙΡΕΙΝ

που απονέμει το Τμήμα Βιοχημείας και Βιοτεχνολογίας του Πανεπι-
στημίου Θεσσαλίας, σε συνεργασία με την Ισπανική spin-off εταιρεία
Nanovex Biotechnologies S.L.

Εγκρίθηκε την από την τριμελή εξεταστική
επιτροπή:

ΤΡΙΜΕΛΗΣ ΕΠΙΤΡΟΠΗ

ΟΝΟΜΑΤΕΠΩΝΥΜΟ

ΒΑΘΜΙΔΑ

ΥΠΟΓΡΑΦΗ

(Επιβλέπων/ουσα)

(Μέλος 1)

(Μέλος 2)

INDEX

| | |
|---|----------|
| ABSTRACT..... | I |
| 1. INTRODUCTION..... | 2 |
| 1.1. Niosomes..... | 3 |
| 1.2. Resveratrol..... | 4 |
| 1.3. Sodium Diclofenac..... | 5 |
| 1.4. Encapsulation methods | 6 |
| 1.4.1 FACTORS AFFECTING NIOSOMES FORMATION..... | 11 |
| 1.4.1.1. Formulation aspects..... | 11 |
| A. Non-ionic surfactant structure..... | 11 |
| B. Additives..... | 13 |
| C. Encapsulated substance..... | 14 |
| 1.4.1.2. Surfactant concentration..... | 14 |
| 1.4.1.3. Temperature..... | 15 |
| 1.4.2. NIOSOMES PREPARATION METHOD..... | 15 |
| 1.4.2.1. Agitation-Sonication..... | 15 |
| 1.4.2.2. Thin Film Hydration (TFH)..... | 16 |
| 1.4.2.3. Dehydration – Rehydration Vesicles (DRV) method..... | 17 |
| 1.4.2.4. Reverse Phase Evaporation (REV)..... | 18 |
| 1.4.2.5. Ether Injection Method (EIM)..... | 18 |
| 1.4.3. NIOSOME CHARACTERIZATION..... | 19 |
| 1.4.3.1 Vesicle mean size and size distribution..... | 19 |
| 1.4.3.2 Zeta potential (ζ -potential)..... | 19 |
| 1.4.3.3. Stability..... | 20 |
| 1.4.3.4. Morphology..... | 20 |
| 1.4.3.5. Entrapment efficiency..... | 20 |
| 1.4.4. NIOSOMES APPLICATIONS..... | 21 |

| | |
|---|-----------|
| 1.4.4.1. Dermal and transdermal delivery..... | 21 |
| 1.4.4.2. Oral delivery..... | 22 |
| 1.4.4.3. Pulmonary delivery..... | 22 |
| 1.4.4.4. Ocular delivery..... | 23 |
| 1.4.4.5. Parenteral delivery..... | 23 |
| 1.4.4.6. Gene delivery..... | 23 |
| 1.4.4.7. Therapeutics/Diagnosis..... | 23 |
| 2. Objectives..... | 25 |
| 3. Conclusion..... | 27 |
| 4. References..... | 29 |

ABSTRACT

This work concerns the current methodology for the generation of vesicles by the self-synthesis of non-ionic surfactants (**niosomes**) and their application in the pharmaceutical, food and cosmetic industries. The suitability of the niosomes for encapsulation of bioactive compounds is reported. For this purpose, a two-stage technique is used to create the niosomes, the first stage is mechanical agitation and the second is the sonication. Methods are also presented for evaluating the formation of the niosomes. We describe the method of incorporating bioactive compounds into liposomes and *ex-vivo* percutaneous absorption by considering the role of vesicular systems as nanocarriers in dermal and transdermal administration. Finally, reference is made to the methodology for assessing the effect of vesicle composition on its stability and its physicochemical properties.

Keywords: Non-ionic surfactant; Stability; Entrapment Efficiency; Thin Film Hydration Method, Functional food

1. INTRODUCTION

1.1 Niosomes

The formation of the niosomes results from self-synthesis of non-ionic surfactants that result in closed two-layered structures (vesicles). As surfactants we call the amphiphilic molecules having two distinct regions formed by a very small attraction structural group for the solvent called the tail (hydrophobic part) and another strong pulling group for the solvent called the head (hydrophilic part). The hydrophilic group, depending on its nature, can be used to classify the surfactants as ionic or nonionic in which the surfactant has no apparent ionic charge.

The first report of nonionic surfactants is found in cosmetic applications by L'Oreal, where nonionic surfactants were preferred due to their lower irritant power (Mahale, et al., 2012).

The result of self-synthesis is that the hydrophilic head groups lean towards the aqueous medium to have maximum contact therewith and the hydrophobic portions of the surfactant are preserved protected by this aqueous medium. Figure 2 shows the schematic structure of a niosome.

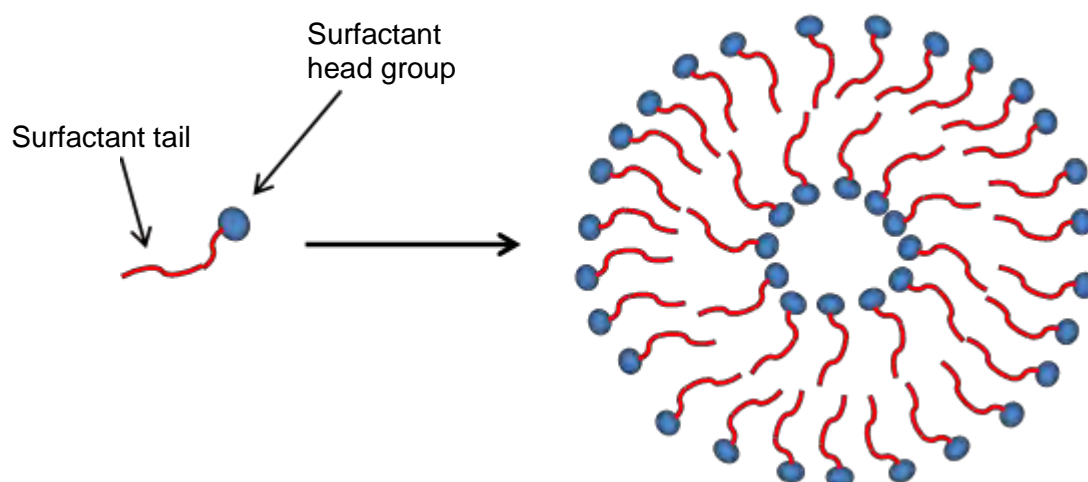


Fig. 1. Schematic structure of a niosome

1.2 Resveratrol

Resveratrol (3,5,4'- trihydroxy-trans-stilbene; RSV) is a natural polyphenol from the stilbenes family found in a wide variety of plants used as dietary supplement, nutraceutical ingredient, and also in topical use products.

Extraction methods can be used to acquire RSV either 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. Thus, RSV has been shown to display anticancer activity (Karthikeyan, *et al.*, 2013; Surh, *et al.*, 1999), and prolong the lifespan (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 from 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). Similarly, RSV contributes to 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 were carried out *in vitro*. Some authors do not accept the equivalence *in vitro* – *in vivo* to RSV benefits due to its poor bioavailability, which is due to its low stability, increased oxidation, and low solubility in water (Gambini, *et al.*, 2013; Ndiaye, *et al.*, 2011). Moreover, RSV is a photosensitive molecule existing in the form of *cis* and *trans* structural isomers (Fig. 1), in which the aforementioned biological effects are demonstrated. The exposure to light of RSV for one hour (Vian, *et al.*, 2005) results in 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.

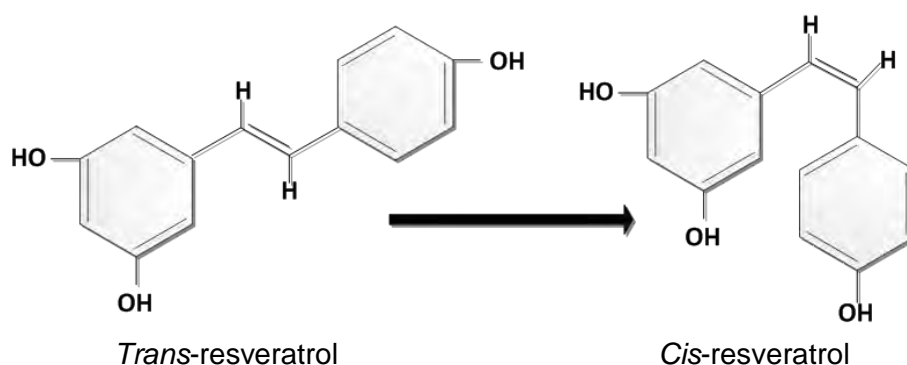


Fig. 2. Isomers of resveratrol

To exert a significant effect on cellular and molecular levels, RSV must penetrate the cells. There is a reduction in accessibility to RSV cells due to many events involved in multiple stages of the penetration process, mainly complexation with extracellular molecules, such as serum proteins, fatty acids or lipoproteins. For this reason, encapsulation of RSV serves to improve its penetration and release into cells (Delmas, et al., 2011; Paolino, et al., 2008).

1.3 Sodium Diclofenac

One of the most common non-steroidal drugs to fight pain and inflammation is diclofenac, which is prescribed. Diclofenac prevents the presence of the cyclooxygenase enzymes COX-1 and COX-2 and inhibits the synthesis of prostaglandins.

Diclofenac given in two salt forms: diclofenac sodium and diclofenac potassium, with sodium salt being the most commonly prescribed (Moore et al., 2011), is indicated in rheumatic diseases, gout, migraine, musculoskeletal and postoperative pain and is also used locally in actinic keratosis, ophthalmically for inflammatory and postoperative inflammation and seasonal allergic conjunctivitis. Diclofenac has been associated with opioid-sparing effects in the treatment of cancer (Dodds et al., 2014, Palmero et al., 1999; Bjorkman et al., 1993).

Diclofenac is orally administered and undergoes first-pass metabolism to a large extent. Plasma proteins bind diclofenac at therapeutic levels with a half-life of 1-2 hours (Davies and Anderson, 1997). Because of its sensitivity to light, Diclofenac sodium (DS) can not be effectively absorbed after transdermal application having gastrointestinal adverse effects (Ioele et al., 2015, Menasse et al., 1978). The composition of DS (Figure 2) consists of two benzene rings and a carboxyl group and is expected to aggregate in the aqueous solution when it has a fairly high concentration because its structure resembles a surfactant. It has been shown that although surfactant DS does not form micelles due to the effect of the counter ion (Ledwidge et al., 1998, Attwood et al., 1983).

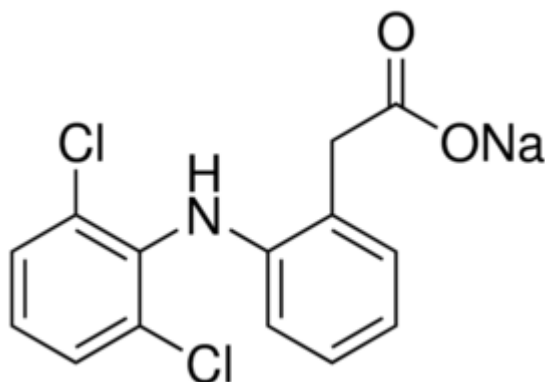


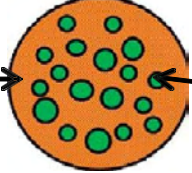
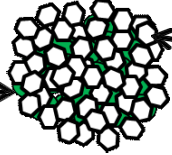
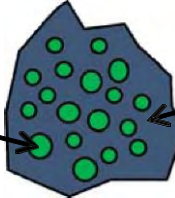
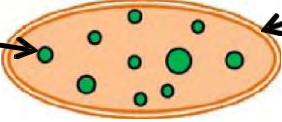
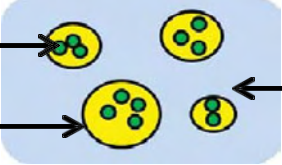
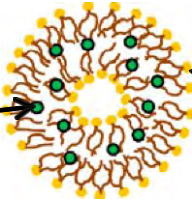
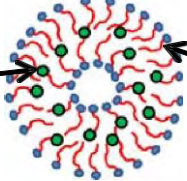
Fig. 3. Molecular structure of diclofenac sodium

1.4 Encapsulation methods

The trapping of a substance (active agent) into another substance (wall material) is called encapsulation (Nedovic, et al., 2011). The protection of RSV from degradation and the effective mitigation of its limitations has been investigated in various encapsulation studies. The most common methods of encapsulating drugs or bioactive compounds are described in Table 1.

- **Spray-drying.** The spray-drying method of encapsulation which produces spherical particles with diameters ranging from 10 to 100 μm has been used in the food industry since 1950 allowing the entrapment of a variety of compounds due to its economic, flexible and continuous operation. The main problem with this method is the shortage of soluble in water shell materials used for the production of the particle matrix (Desai and Jin Park, 2005; Fang and Bhandari, 2010).
- **Co-crystallization.** Another method of encapsulation is the Co-crystallization by which the crystalline structure of sucrose is modified from a perfect to an irregular agglomerated crystal at high temperatures (120 $^{\circ}\text{C}$) thus providing a porous matrix for the incorporation of a second active compound at the same time. This enables its incorporation into the void spaces inside the agglomerates of the micro crystals, with a size less than 30 μm (Bhandari, et al., 1998; Fang and Bhandari, 2010).

Table 1. Schematic illustration of the main encapsulation methods of drugs or bioactive compounds (Nedovic *et al.*, 2002 ; Bhandari *et al.*, 1998).

| Method | Illustration of characteristics |
|----------------------------|--|
| Spray drying |  <p>Matrix</p> <p>Bioactive compound</p> |
| Co-crystallization |  <p>Bioactive compound</p> <p>Sugar crystals</p> |
| Freeze drying |  <p>Bioactive compound</p> <p>Matrix</p> |
| Yeast encapsulation |  <p>Bioactive compound</p> <p>Yeast cell</p> |
| Emulsification |  <p>Bioactive compound</p> <p>Water</p> <p>Oil droplet</p> |
| Vesicle entrapment |  <p>Bioactive compound</p> <p>Phospholipid</p> |
| |  <p>Bioactive compound</p> <p>Surfactant</p> |

This method produces final encapsulated materials that are stable, homogeneous and of high solubility (Beristain, et al., 1996).

- **Freeze-drying**, which works by freezing the material and then reducing the pressure is mainly used for the encapsulation of heat sensitive and water-soluble compounds (Desai and Jin Park, 2005). This method aims to make the frozen water in the material directly extracted from the solid phase into the gas phase. With this technique, the particles obtained have indeterminate forms and consist of a matrix containing the active compounds of interest (Fang and Bhandari, 2010).
- **Yeast encapsulation**. Yeast encapsulation by which cells are grown in liquid culture medium has many benefits over other microencapsulation technologies due to the structure of yeast cell wall and its natural properties (Nelson, 2002). In this process an aqueous suspension consisting of yeast cells and the compound to be encapsulated is mixed allowing the compound to pass freely through the cell wall and the membrane, and to remain passively within the cell. (Bishop, et al., 1998). This method is preferred for the entrapment of polyphenols, including RSV, although it presents low entrapment efficiency values (~15%) (Shi, et al., 2008).
- **Emulsification**. An emulsion is comprised by two immiscible liquids of different composition, a droplet-shaped liquid dispersed in the second. In food systems, the droplet diameter ranges from 0.1 to 100 μm .

In the case where the dispersed droplets contain smaller droplets of a different phase, we have a ternary system called multiple emulsion and has either the oil-in-water (W1 / O / W2) or oil-in-water (O1 / W / O2) structure. The encapsulation of the trans-resveratrol with the use of W1/O/W2 double emulsions has 40 % efficiency. (Hemar, et al., 2010, Matos, et al., 2014).

- **Vesicle entrapment.** Another entrapment method is the Vesicle entrapment which is used for the encapsulation of hydrophilic compounds into the aqueous compartments between the bilayers and lipophilic compounds inside the bilayer matrix (Saini, et al., 2011; Uchegbu and Vyas, 1998). for either food, pharmaceutical or cosmetic applications, and also can work as drug carriers. The Vesicles are colloidal particles in which a concentric bilayer made up of amphiphilic molecules surrounds an aqueous compartment. The most common vesicles are liposomes and niosomes
- **Liposomes** are essentially spherical bilayer vesicles formed by phospholipid self-synthesis based on the unfavorable interactions occurring between phospholipids and water molecules in which polar head groups of phospholipids are exposed to the aqueous phases (internal and external) and hydrophobic hydrocarbon tails are forced to face each other in a bilayer (Da Silva Malheiros, et al., 2010). Liposomes were firstly mentioned by Bangham (Bangham, et al., 1965)

Liposomes can be used for the encapsulation, delivery, and the controlled release of hydrophilic, lipophilic, and amphiphilic compounds because their structure has lipid and aqueous phases (Da Silva Malheiros, et al., 2010; Du Plessis, et al., 1994).

Niosomes ranging from 10 nm to 3 µm (Moghassemi and Hadjizadeh, 2014) are vesicles created by the self-assembly of non-ionic surfactants in aqueous media that lead to closed bilayer structures (Uchegbu and Vyas, 1998). As liposomes, they are created by the adverse interactions between surfactants and water molecules and can trap hydrophilic, lipophilic and amphiphilic compounds (Mahale, et al., 2012, Moghassemi and Hadjizadeh, 2014).

Comparing niosomes and liposomes characteristics (Marianecci, et al., 2014; Uchegbu and Vyas, 1998) we come to the following points:

- Encapsulation of drugs or bioactive compounds into niosomes enhances its bioavailability, while with liposomes there is no enhances its bioavailability.
- 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 and liposomes 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 and liposomes improve skin penetration of drugs or bioactive compounds.
- Variables involved in niosome formation can be easily controlled.
- Niosomes and liposomes 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.

1.4.1 FACTORS AFFECTING NIOSOMES FORMATION

The production of niosomes from the surfactants is regulated by certain parameters. These are mentioned below:

1.4.1.1 Formulation aspects

The comprehension of the role that the key ingredients have in the formation of the niosomal structure before it is prepared is important

A. Structure of non – ionic surfactant

Niosome formation depends on the structure of the non – ionic surfactants. This can be seen because despite the fact that the formation of a bilayer membrane in aqueous media usually needs a surfactant with higher contribution of the hydrophobic part we can create niosomes from hydrophilic surfactants by also using an optimum level of hydrophobic membrane stabilizer (e.g. Cholesterol). (Manconi, *et al.*, 2006).

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

One indicator of the ability of a surfactant to form vesicle is the HLB (Hydrophilic – Lipophilic Balance) because each surfactant molecule is consisted by hydrophilic and lipophilic groups and the ratio between the weight percentages of these two groups for non-ionic surfactants determines their behavior (Griffin, 1955). So a value of 1 for HLB indicates a lipophilic surfactant and a a value of 20 a completely hydrophilic molecule. The relationship between HLB values and surfactant application is shown in table 2.

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 |

Niosomes are formed usually when HLB values range between 4 and 8 (Saini, *et al.*, 2011), but niosomes can be produced also by surfactants that have HLB values outside of this range.

Another indicator of the ability of a surfactant to form niosomes is the CPP (*Critical Packing Parameter*) which 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 volume of the hydrophobic group, l_c is the critical length of the hydrophobic group, and a_0 is the area of the head of the hydrophilic group. When CPP value is between 1/2 and 1 the surfactant can be used for the formation of vesicles, when CPP value is lower than 1/3 the surfactant give spherical micelles, for values from 1/3 to 1/2 cylindrical micelles are formed, for values around 1 surfactant form planar bilayers, and for values higher than 1 they form inverted micelles (Israelachvili, 2011).

B. Additives

A common practice in the production of high stability vesicles is the use of additives in niosome formulation. These additives can be classified by the area that their functionality affects, to the following:

- **Membrane additives.** Cholesterol which improves the main vesicular properties, such as entrapment efficiency, release, and stability under storage (Biswal, *et al.*, 2008; Shilpa, *et al.*, 2011).is the most common additive of this category. Cholesterol abolishes the gel to liquid phase transition of liposomal and niosomal systems (Manosroi, *et al.*, 2003), by changing the fluidity of the bilayer membrane thus providing greater stability (Kumar and Rajeshwarrao, 2011). Recent reports state that dodecanol could replace cholesterol as a stabilizer in food-grade niosome formulations (Pando, *et al.*, 2015).
- **Surface additives.** This category includes additives that act on the niosome surface. Dicetyl phosphate (DCP) which imparts negative charge on the surface of niosomes which remain stabilized by electrostatic repulsion (Waddad, *et al.*, 2013) is the most common additive of this category.
- **Steric additives.** The most common additive in this category is Polyethylene glycol (PEG) which stabilize niosomes by providing them steric repulsion. Surface-attached PEG chains water solubility and flexibility sterically stabilizes the vesicles 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

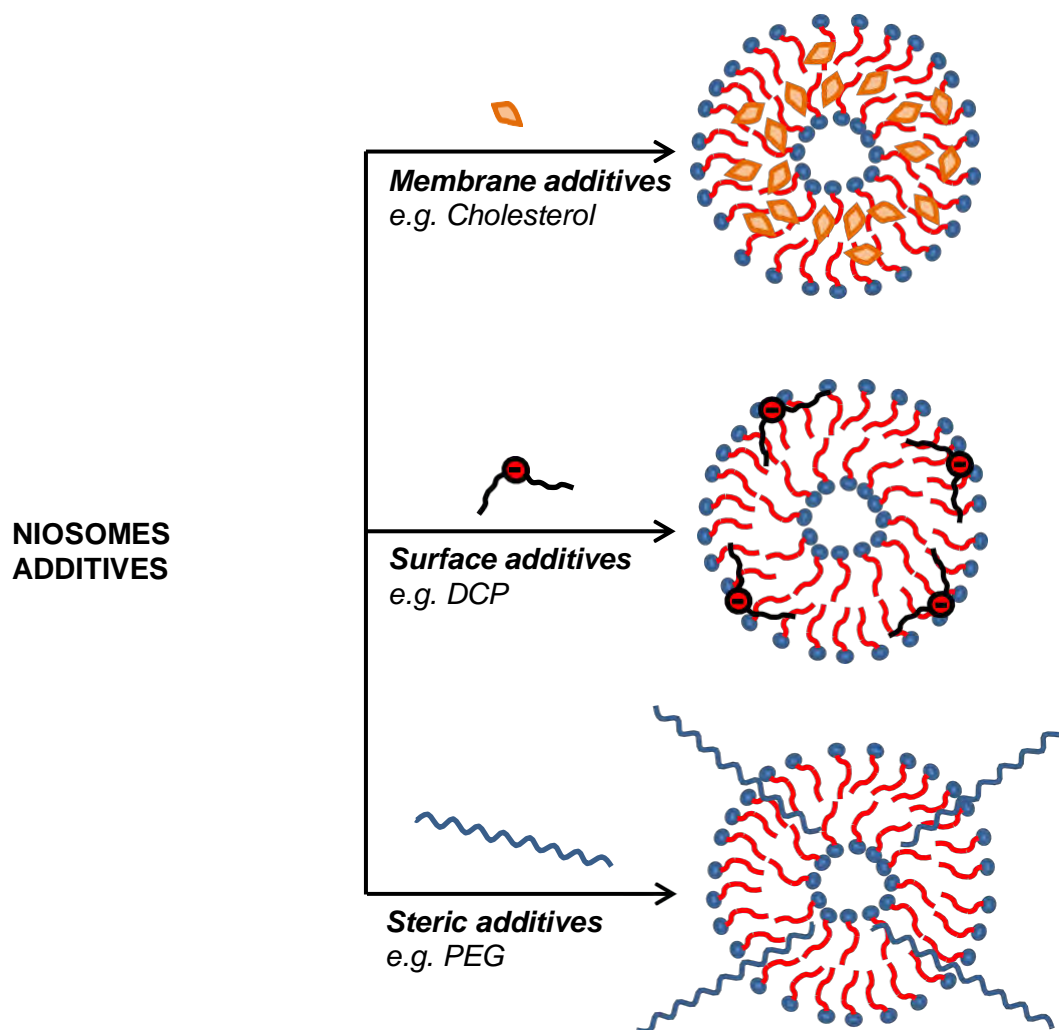


Fig. 3. Types of additives for niosomes (Manosroi, et al., 2003; Waddad, et al., 2013 ; Garbuzenko, et al., 2005; Lasic, 1994)

C. Encapsulated substance

The morphology and stability of the final dispersion can be affected by the substances, either drugs or bioactive compounds that are being incorporated into niosomes, the most problematic are the amphiphilic compounds (Kumar and Rajeshwarao, 2011; Uchegbu and Vyas, 1998). One amphiphilic drug is Doxorubicin which leads to aggregation when it is encapsulated in niosomes. This can be overcome by the addition of a steric stabilizer (Kumar and Rajeshwarao, 2011).

1.4.1.2 Surfactant concentration

The production of niosomes usually demands a 10–30 mM (1–2.5% w/w) concentration of surfactant (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 which leads to an increase in system viscosity (Pando, et al.,

2013a; Uchegbu and Vyas, 1998)

1.4.1.3 Temperature

In the production of niosomes the hydrating temperature should be above the T_c of the system which is the temperature that gels transition to liquids (Kumar and Rajeshwarrao, 2011; Marianecci, *et al.*, 2014). When the hydrating temperature used is above T_c the niosomes show higher entrapment efficiencies than when it is lower than T_c (Biswal, *et al.*, 2008; Hao, *et al.*, 2002; Kumar and Rajeshwarrao, 2011).

1.4.2 NIOSOMES PREPARATION METHODS

During the process of niosomes formation additional energy is required (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.

1.4.2.1 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.

The best operational procedure seems to be an initial sample homogenization by agitation followed by sonication. Sonication produces small unilamellar vesicles (SUVs) with narrow size distribution. This technique involves the aqueous solution containing the compound of interest to be added to the surfactant/stabilizers mixture which is then stirred or sonicated to form niosomes. A schematic diagram of this method is shown in Fig. 4.

Other procedures, such as mechanical agitation, produce multilamellar vesicles (MLV) (Pando, *et al.*, 2013a; Walde and Ichikawa, 2001).

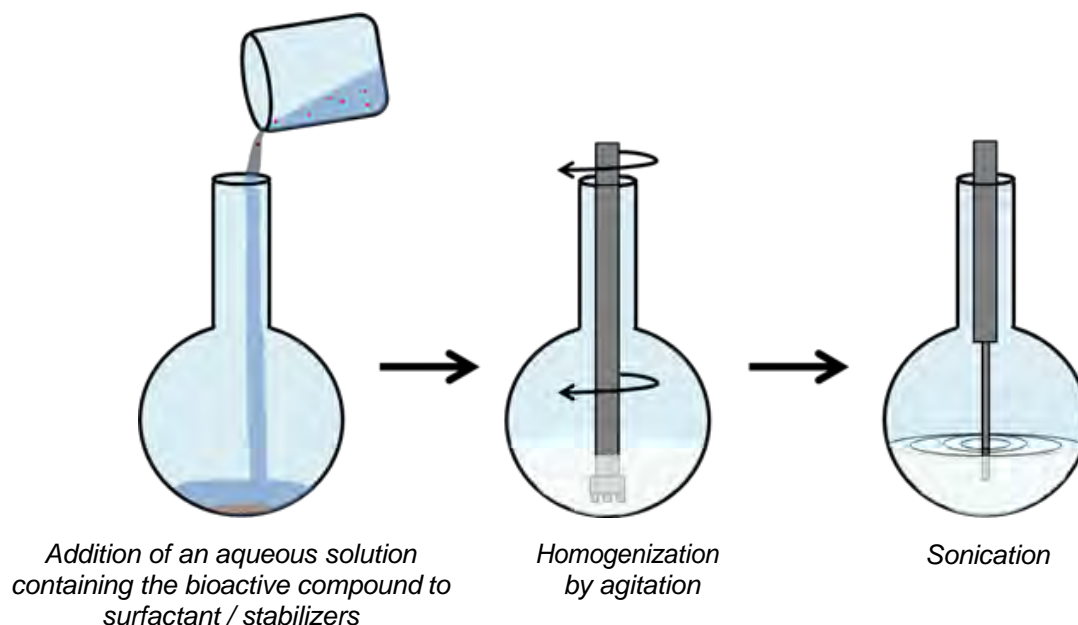


Fig. 4. Agitation – sonication method for niosomes preparation

1.4.2.2 Thin Film Hydration (TFH)

Film Hydration (TFH) described by Bangham *et al.* (Bangham, *et al.*, 1965) is still widely used.

TFH method can be divided into stages. Firstly, the dissolution of the surfactant/stabilizers in an organic solvent, as chloroform or ethanol. Secondly, the removal of the organic solvent by vacuum evaporation. Thirdly, the hydration of the thin film formed on the wall of the flask with an aqueous solution containing the substance of interest. (Hao and Li, 2011; Manconi, *et al.*, 2006).

Finally, 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).

This procedure is shown in Fig. 5.

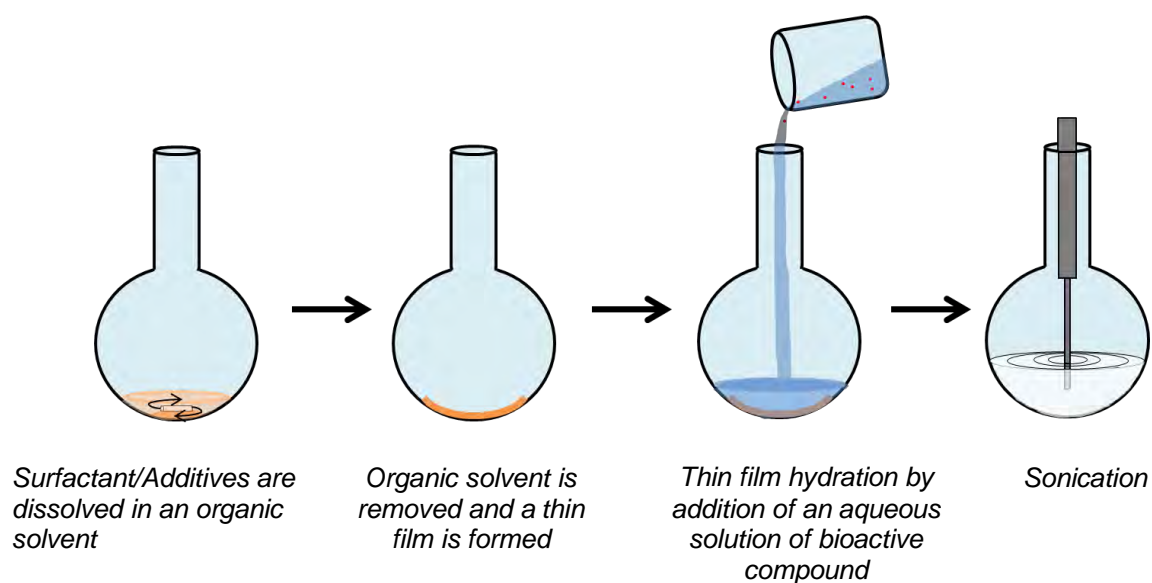


Fig. 5. Thin Film Hydration method for niosomes preparation

1.4.2.3 Dehydration – Rehydration Vesicles (DRV) method

DRV described by Kirby and Gregoriadis (Kirby and Gregoriadis, 1984) can be divided into stages. Firstly, niosomes are obtained by TFH method. Secondly, the niosomes are frozen in liquid nitrogen and freeze-dry overnight to form a cake that contains the bioactive compound/. 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.

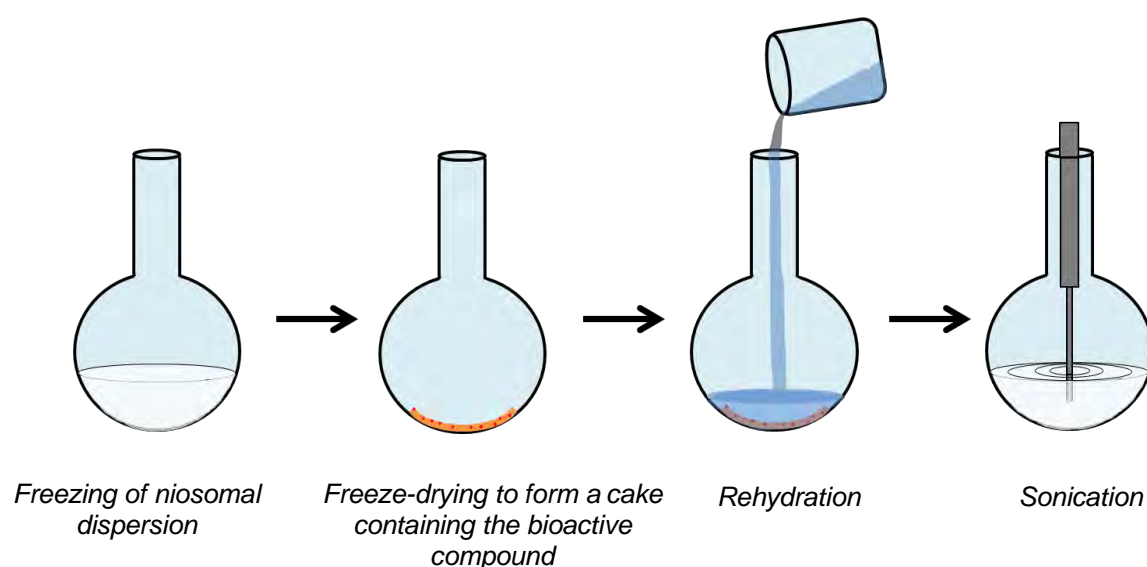


Fig. 6. Dehydration – Rehydration Vesicles method for niosomes preparation

1.4.2.4 Reverse Phase Evaporation (REV)

Before the application of this method, surfactant and additives are previously dissolved in an organic solvent. This method can be divided into the following stages: firstly, the organic solution is added to an aqueous phase, containing the bioactive compound. Secondly, this mixture is sonicated to obtain an emulsion. Finally, the organic solvent is removed in a vacuum rotary evaporator 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.

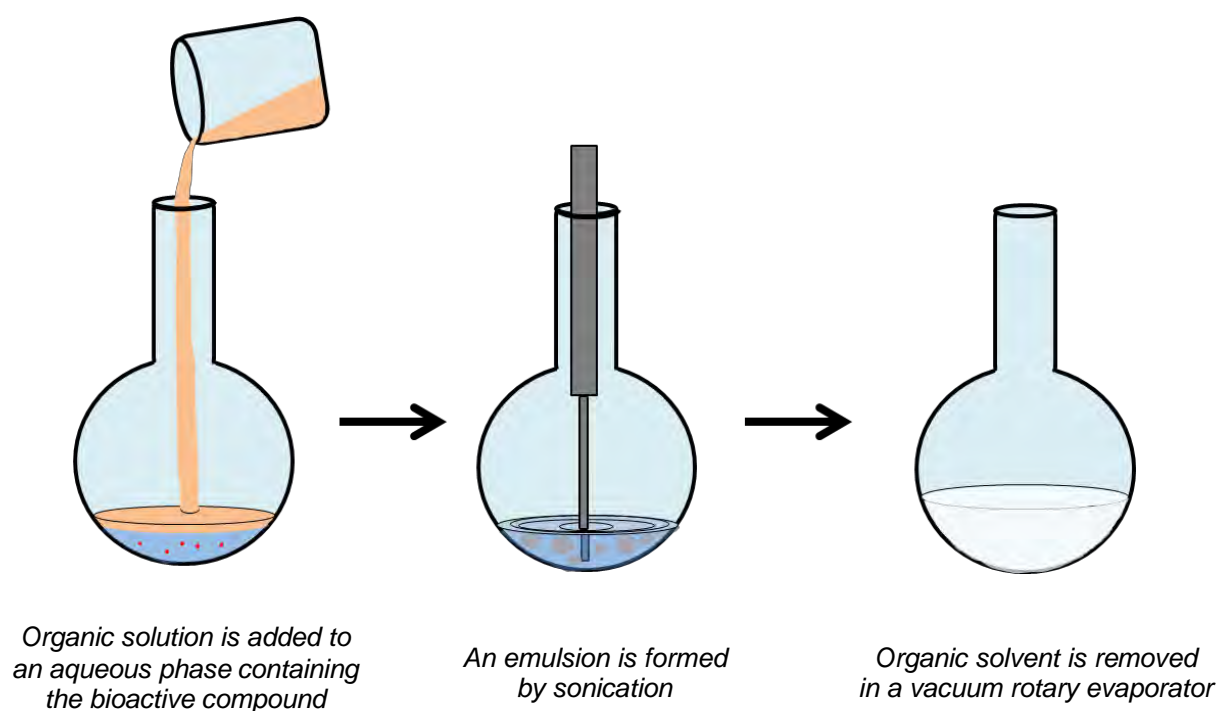


Fig. 7. Reverse Phase Evaporation method for niosomes preparation

1.4.2.5 Ether Injection Method (EIM)

This technique can be divided into two stages. Firstly, the surfactant and additives dissolved in an organic solvent, as diethyl ether or ethanol are injected slowly through a needle into an aqueous phase containing the bioactive compound. Secondly, the organic solvent is removed using a vacuum rotary evaporator and niosomes are formed after organic solvent is removed if diethyl ether 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). This procedure is shown in Fig. 8. When ethanol is used as organic solvent, niosomes are formed during the injection process,

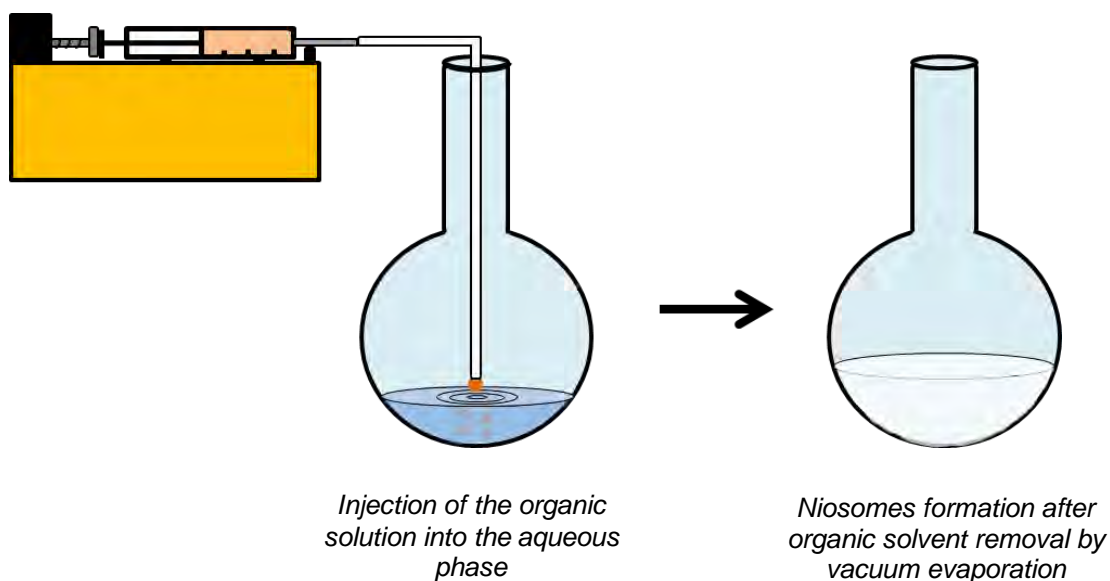


Fig. 8. Ether Injection Method for niosomes preparation

1.4.3 NIOSOMES CHARACTERIZATION

The most important parameters to characterize niosomes are the following:

1.4.3.1 Vesicle mean size and its distribution of size

It is supposed that Niosomes have are spherical, thus a Niosomes size is appointed a mean value (Z-Average), ranging from 20 nm to 50 μm (Mean particle size, diameter, nm) (Moghassemi and Hadjizadeh, 2014). Polydispersity Index (PDI or PI) is the parameter used to characterize the distribution of niosomes size (Pando, *et al.*, 2013b). The 0 value for a PDI dictates vesicles which are totally monodispersed, on the other hand a PDI value of 1 dictates vesicles which are polydispersed to a high extent. In this study a Zetasizer Nano ZS (Malvern Instruments Ltd, UK utilizing the Dynamic Light Scattering (DSL), determined both the mean (Z-Average) size and PDI of niosomes

1.4.3.2 Zeta potential (ζ -potential)

The plane of shear, located between the compact and diffuse layers has an electric potential which is described by this parameter. This means that the ζ -potential is the difference in electric potential that the dispersion medium has in relation to the corresponding one of the stationary layer of fluid which is attached to the dispersed particle (Clogston and Patri, 2011). The ζ -potential indicates the level of niosomes stability thus, a high absolute value means electrostatic repulsion between vesicles leading to high stability, since it prevents aggregation and fusion of niosomes (Pando, *et al.*, 2013b).

1.4.3.3 Stability

The encapsulated compound presents aggregation, fusion, and leakage problems in reference with vesicles storage. For this reason backscattering (BS) profiles were measured to monitor niosomes stability with time. This was done in a Turbiscan Lab[®] Expert apparatus (Formulation, France) by incorporating an Ageing Station (Formulation, France). The produced findings present macroscopically the niosomes at a given time showing the changes in the distribution of the vesicles size and/or appearance of a creaming layer or a clarification front with time (Pando, *et al.*, 2013a).

1.4.3.4 Morphology

Negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM (Japan) was used for the analysis of the structure of niosomes and the confirmation of vesicles morphology.

1.4.3.5 Entrapment efficiency

The percentage of the entrapped bioactive compound or drug into purified niosomes gives the efficiency of Encapsulation (EE%). The measurement of the difference between entrapped and total amount of drug or bioactive compound initially incorporated determines this percentage. This also refers to the total amount of bioactive compound which is present in a non-purified sample. Different analytical techniques must be used depending on compound encapsulated. In this work, bioactive compounds were determined by chromatography (HP series 1100 chromatograph, Hewlett Packard, USA) using UV/VIS absorbance and fluorescence detectors.

The various methods for the characterization of niosomes is presented in Table 3

Table 3. 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 |

1.4.4 NIOSOMES APPLICATIONS

The high entrapment efficiency of the niosomes for different types of drugs or bioactive compounds was the main reason for their use in cosmetic industry and by pharmaceutical companies (Shilpa, *et al.*, 2011).

Thus, niosomes can be used as nanocarriers in several delivery systems, such as: gene delivery, anti-bacterial, antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-malarial, anti- asthma, anti-amyloid, anti-Alzheimer, anti- fungal, nutraceuticals, etc. (Moghassemi and Hadjizadeh, 2014).

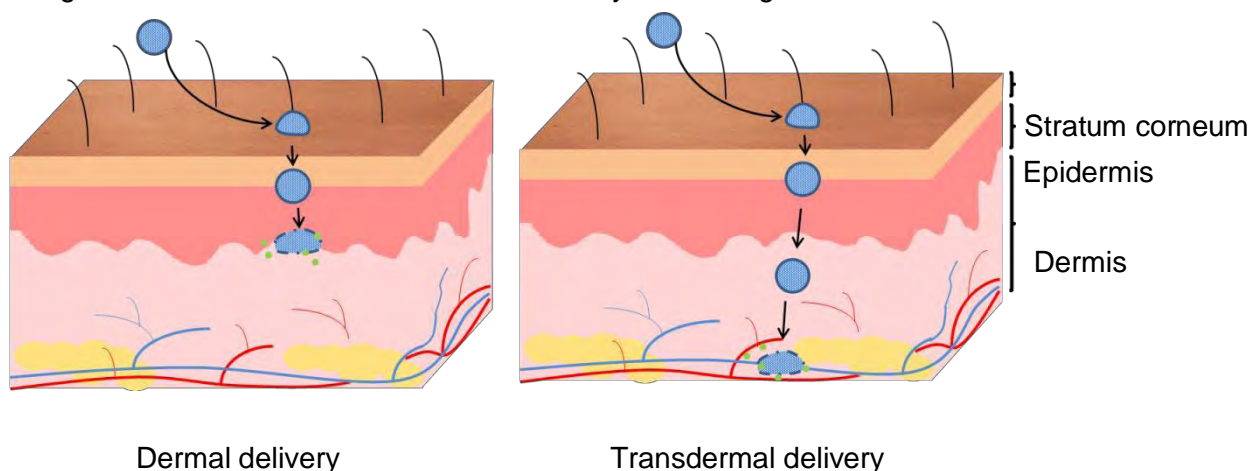
1.4.4.1 Dermal and transdermal delivery

Niosomes in a drug or bioactive compound applied dermally into the skin can increase the residence time of the drug in skin deeper layers. This topical application is used for the treatment of diseases or for other health benefits. The resulting higher concentrations in epidermis and dermis reduces the systemic absorption of the drug thus increases 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 and has several advantages, such as the avoidance

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

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



1.4.4.2 Oral delivery

Oral administration of drugs is the most common system of delivery and there is an increase in the use of functional foods and nutraceuticals because of their health gains, (Shahidi, 2009).

Niosomes encapsulation is used to address the bioavailability problems of orally administered compounds that are the result of poor solubility, low dissolution rate, degradation, and unpredictable absorption, and protects the compound and increases its bioavailability (Gurrapu, *et al.*, 2012; Pando, *et al.*, 2013a) and also help to mask undesirable flavors (Tavano, *et al.*, 2014).

1.4.4.3 Pulmonary delivery

Pulmonary delivery because of the high drug concentration located at the specific site of action is preferred in inflammatory diseases such as infections or cancer of the respiratory tract.

Because of the inability of some lipophilic drugs to permeate through the hydrophilic mucus to reach a site of action a targeted delivery using niosomes is used for the drug to reach this specific location thus having the optimal effect for the patient, (Marianecchi, *et al.*, 2014; Terzano, *et al.*, 2005).

Nebulizers are the most common aerosol delivery technologies because vesicular structures may be delivered without further processing (Saari, *et al.*, 1999).

1.4.4.4 Ocular delivery

The implementation of the ocular delivery of drugs presents some difficulties, both anatomical and physiological. A way to overcome these is the use of carriers (Marianecci, *et al.*, 2014). In several cases the controlled delivery of ophthalmic drugs improves their ocular bioavailability compared to traditional eye drops, (Abdelbary and El-Gendy, 2008; Di Colo and Zambito, 2002).

The use of niosomal systems as nanocarriers present some advantages such as the delivery of the drug at the corneal surface for a prolonged time in a controlled way, and the prevention by the tear/corneal epithelial surface enzymes of drug metabolism, (Abdelbary and El-Gendy, 2008).

1.4.4.5 Parenteral delivery

Drugs or bioactive compounds are most commonly administered by the parenteral route. This requires frequent injections for the maintenance of an effective concentration of the drug. The problems of parenteral administration have been overcome by the development of vesicles because they provide a predictable targeted and sustained release of the drug (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).

1.4.4.6 Gene delivery

Niosomes can be applied for topical gene delivery in gene therapies for the treatment of different inherited or acquired disorders. Contrary to traditional forms of therapy, gene therapies with the application of niosomes use genetic material such as DNA, oligonucleotides, small interfering RNAs, ribozymes, DNazymes and are painless and have an easy administration, (Mahale, *et al.*, 2012).

Gene therapy has the theoretical potential to treat almost any disease (Marianecci, *et al.*, 2014).

1.4.4.7 Therapeutics / Diagnostics

The use of vesicles as nanocarriers is a successful way to achieve an enhanced targeted delivery into specific cells using antibody/antigen recognition (Simard and Leroux, 2009). Nowadays due to advancements in technology mainly focused on liposomes, several vaccines with adjuvants based on vesicles have reached the final stages of clinical evaluation or have cleared for use, (Watson, *et al.*, 2012).

Niosomes can be used for these purposes. In addition, niosomes increase the chances for early diagnosis with their use as imaging agents for tumors or for making biosensors to detect diseases, (Uchegbu and Vyas, 1998; Yu, *et al.*, 2011).

2. OBJECTIVES

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

- The description of the method for the preparation of niosomes by a two-stage technique: thin film hydration method, mechanical agitation and sonication. The presentation of the method for the determination of the operation parameters influence on the mean size and the distribution, size, stability and entrapment efficiency of vesicle.
- The presentation of the method for the encapsulation into liposomes for topical use.
- The presentation of the method for the preparation of entrapped DS and the implementation of variance analysis (ANOVA) for the optimization of the key parameters involved in the preparation of niosomes.

3. CONCLUSIONS

Research has shown the excellent potential for the use of liposomes and niosomes nanoparticles in encapsulation of hydrophobic and hydrophilic bioactives compounds. The physicochemical characteristics, as well as safety, stability and release profiles of the liposomes and niosomes formulations will determine the manner of their behavior in vivo and usefulness for human health. Compared to liposomes, niosomes have the advantage that the components are extremely cheap compared to phospholipids and both the lipids and non-ionic surfactants are similarly stable. A disadvantage is that the currently commercially available surfactants (Spans and Tweens) are polydisperse. At the first place niosomes have been used in cosmetic industry and then have come to the attention of the pharmaceutical companies. Research on the production of niosomes in the food industry has not gone far enough. The use of the niosomes could be utilized in the manufacture of nutraceuticals. Further studies demonstrating the synergistic health benefits in human or animal models will help to generate concrete experimental data and establish the technique in food industry. Regarding niosomes in cosmetic industry were shown to improve both dermal and transdermal drug delivery in comparison with conventional liposomes.

4. REFERENCES

4. References

- Abdelbary, G., El-gendy, N. (2008). Niosome-encapsulated gentamicin for ophthalmic controlled delivery. *AAPS PharmSciTech*, 9(3), 740-747.
- Abdelkader, H., Ismail, S., Kamal, A., Alany, R. G. (2011). Design and evaluation of controlled-release niosomes and discomes for naltrexone hydrochloride ocular delivery. *Journal of Pharmaceutical Sciences*, 100(5), 1833-1846.
- Aditya, N. P., Aditya, S., Yang, H., Kim, H. W., Park, S. O., Ko, S. (2015a). Co-delivery of hydrophobic curcumin and hydrophilic catechin by water-in-oil-in-water double emulsion. *Food Chemistry*, 173: 7-13.
- Attwood, D. Florence, A.T., *Surfactant Systems* (1983): Their Chemistry, Pharmacy and Biology, Chapman & Hall, London (U.K).
- Bangham, A., Standish, M. M., Watkins, J. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, 13(1), 238-IN227.
- Beristain, C. I., Vazquez, A., Garcia, H. S., Vernon-Carter, E. J. (1996). Encapsulation of orange peel oil by co-crystallization. *LWT - Food Science and Technology*, 29(7), 645-647.
- Bhandari, B. R., Datta, N., D'Arcy, B. R., Rintoul, G. B. (1998). Co-crystallization of honey with sucrose. *LWT-Food Science and Technology*, 31(2), 138-142.
- Bishop, J., Nelson, G., Lamb, J. (1998). Microencapsulation in yeast cells. *Journal of Microencapsulation*, 15(6), 761-773.
- Biswal, S., Murthy, P., Sahu, J., Sahoo, P., Amir, F. (2008). Vesicles of non-ionic surfactants (niosomes) and drug delivery potential. *International Journal of Pharmaceuticals Science and Nanotechnology*, 1(1), 1-8.
- Björkhem, I., Heverin, M., Leoni, V., Meaney, S., Diczfalusy, U. (2006). Oxysterols and Alzheimer's disease. *Acta Neurologica Scandinavica*, 114, 43-49.
- Chen, Q., McGillivray, D., Wen, J., Zhong, F., Quek, S. Y. (2013a). Co-encapsulation of fish oil with phytosterol esters and limonene by milk proteins. *Journal of Food Engineering*, 117: 505-512.
- Clogston, J. D., Patri, A. K. (2011). Zeta potential measurement. *Springer Protocols: Characterization of Nanoparticles Intended for Drug Delivery*, in Humana Press (Hertfordshire, UK)
- Coradini, K., Lima, F. O., Oliveira, C. M., Chaves, P. S., Athayde, M. L., Carvalho, L. M., Beck, R. C. R. (2014). Co-encapsulation of resveratrol and curcumin in lipid-core nanocapsules improves their in vitro antioxidant effects. *European Journal of Pharmaceutics and Biopharmaceutics*, 88(1), 178-185.
- Da Silva Malheiros, P., Daroit, D. J., Brandelli, A. (2010). Food applications of liposome-encapsulated antimicrobial peptides. *Trends in Food Science & Technology*, 21(6), 284-292.
- Dasgupta, S., Bandyopadhyay, M. (2013). Neuroprotective mode of action of resveratrol in central nervous system. *PharmaNutrition*, 1(3), 90-97.

- Delmas, D., Aires, V., Limagne, E., Dutartre, P., Mazué, F., Ghiringhelli, F., Latruffe, N. (2011). Transport, stability, and biological activity of resveratrol. *Annals of the New York Academy of Sciences*, 1215(1), 48-59.
- Desai, K. G. H., Jin Park, H. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology*, 23(7), 1361-1394.
- Di Colo, G., Zambito, Y. (2002). A study of release mechanisms of different ophthalmic drugs from erodible ocular inserts based on poly(ethylene oxide). *European Journal of Pharmaceutics and Biopharmaceutics*, 54(2), 193-199.
- Du Plessis, J., Weiner, N., Müller, D. G. (1994). The influence of in vivo treatment of skin with liposomes on the topical absorption of a hydrophilic and a hydrophobic drug in vitro. *International Journal of Pharmaceutics*, 103(3), 277-282
- Eratte, D., McKnight, S., Gengenbach, T. R., Dowling, K., Barrow, C. J., Adhikari, B. P. (2015). Co-encapsulation and characterization of omega-3 fatty acids and probiotic bacteria in whey protein isolate-gum Arabic complex coacervates. *Journal of Functional Foods*, 19: 882-892.
- Fang, Z., Bhandari, B. (2010). Encapsulation of polyphenols – a review. *Trends in Food Science & Technology*, 21(10), 510-523.
- Fini, A., Bassini, G., Monastero, A., Cavallari, C. (2012). *Pharmaceutics* 4: 413.
- Flower, D. R., Perrie, Y. (2013). Immunomic Discovery of Adjuvants and Candidate Subunit Vaccines, in Springer (New York, USA)
- Frémont, L., Belguendouz, L., Delpal, S. (1999). Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life Sciences*, 64(26), 2511-2521.
- Frezza, R. L., Bernardi, A., Hoppe, J. B., Meneghetti, A. B., Matté, A., Battastini, A. M., Pohlmann, A. R., Guterres, S. S., Salbego, C. (2013). Neuroprotective effects of resveratrol against A β administration in rats are improved by lipid-core nanocapsules. *Molecular neurobiology*, 47(3), 1066-1080.
- Gambini, J., López-Grueso, R., Olaso-González, G., Inglés, M., Abdelazid, K., El Alami, M., Bonet-Costa, V., Borrás, C., Viña, J. (2013). Resveratrol: distribución, propiedades y perspectivas. *Revista Española de Geriatria y Gerontología*, 48(2), 79-88
- Garbuzenko, O., Barenholz, Y., Prie, A. (2005). Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chemistry and Physics of Lipids*, 135(2), 117-129.
- Gaudreau, H., Champagne, C. P., Remondetto, G. E., Gomaa, A., Subirade, M. (2016). Co-encapsulation of *Lactobacillus helveticus* cells and green tea extract: Influence on cell survival in simulated gastrointestinal conditions. *Journal of Functional Foods*, 26:451-459.
- Griffin, W. C. (1955). Calculation of HLB values of non-ionic surfactants. *Am Perfumer Essent Oil Rev*, 65, 26-29.
- Guinedi, A. S., Mortada, N. D., Mansour, S., Hathout, R. M. (2005). Preparation and evaluation of reverse phase evaporation and multilamellar niosomes as ophthalmic

carriers of acetazolamide. *International Journal of Pharmaceutics*, 306(1), 71-82.

Gurrapu, A., Jukanti, R., Bobbala, S. R., Kanuganti, S., Jeevana, J. B. (2012). Improved oral delivery of valsartan from maltodextrin based proniosome powders. *Advanced Powder Technology*, 23(5), 583-590.

Hao, Y.-M., Li, K. a. (2011). Entrapment and release difference resulting from hydrogen bonding interactions in niosome. *International Journal of Pharmaceutics*, 403(1–2), 245-253.

Hao, Y., Zhao, F., Li, N., Yang, Y., Li, K. a. (2002). Studies on a high encapsulation of colchicine by a niosome system. *International Journal of Pharmaceutics*, 244(1–2), 73-80.

Hemar, Y., Cheng, L. J., Oliver, C. M., Sanguansri, L., Augustin, M. (2010). Encapsulation of resveratrol using water-in-oil-in-water double emulsions. *Food Biophysics*, 5(2), 120-127.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L.-L. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature*, 425(6954), 191-196.

Hung, L.-M., Chen, J.-K., Huang, S.-S., Lee, R.-S., Su, M.-J. (2000). Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. *Cardiovascular Research*, 47(3), 549-555.

Israelachvili, J. N. (2011). Intermolecular and Surface Forces. 3th Edition, in Academic Press (Madison, USA).

Kamezaki, C., et al. (2016). Synergistic antioxidative effect of astaxanthin and tocotrienol by co-encapsulated in liposomes. *Journal of Clinical Biochemistry and Nutrition*, 59:100-106.

Karthikeyan, S., Rajendra Prasad, N., Ganamani, A., Balamurugan, E. (2013). Anticancer activity of resveratrol-loaded gelatin nanoparticles on NCI-H460 non-small cell lung cancer cells. *Biomedicine & Preventive Nutrition*, 3(1), 64-73.

Kawano, K., Takayama, K., Nagai, T., Maitani, Y. (2003). Preparation and pharmacokinetics of pirarubicin loaded dehydration–rehydration vesicles. *International Journal of Pharmaceutics*, 252(1–2), 73-79.

Kirby, C., Gregoriadis, G. (1984). Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. *Nature Biotechnology*, 2(11), 979-98.

Kristl, J., Teskač, K., Caddeo, C., Abramović, Z., Šentjurc, M. (2009). Improvements of cellular stress response on resveratrol in liposomes. *European Journal of Pharmaceutics and Biopharmaceutics*, 73(2), 253-259.

Kumar, G. P., Rajeshwarrao, P. (2011). Nonionic surfactant vesicular systems for effective drug delivery—an overview. *Acta Pharmaceutica Sinica B*, 1(4), 208-219.

Lantto, T. A., Colucci, M., Zavadová, V., Hiltunen, R., Raasmaja, A. (2009). Cytotoxicity of curcumin, resveratrol and plant extracts from basil, juniper, laurel and parsley in SH-SY5Y and CV1-P cells. *Food Chemistry*, 117(3), 405-411.

Lasic, D. (1990). On the thermodynamic stability of liposomes. *Journal of Colloid and Interface Science*, 140(1), 302-304.

Lasic, D. D. (1994). Sterically stabilized vesicles. *Angewandte Chemie International Edition*, 33(17), 1685-1698.

Lawrence, M., Chauhan, S., Lawrence, S., Barlow, D. (1996). The formation, characterization and stability of non-ionic surfactant vesicles. *STP Pharma Sciences*, 6(1), 49-60.

Ledwidge, M.T., Corrigan, O.I. (1998), *International Journal Pharmacy*. 174:187.

Liu, T., Guo, R. (2007). Structure and transformation of the niosome prepared from PEG 6000/Tween 80/Span 80/H₂O lamellar liquid crystal. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 295(1–3), 130-134.

Lu, X., Ji, C., Xu, H., Li, X., Ding, H., Ye, M., Zhu, Z., Ding, D., Jiang, X., Ding, X. (2009). Resveratrol-loaded polymeric micelles protect cells from A β -induced oxidative stress. *International Journal of Pharmaceutics*, 375(1), 89-96.

Magalhães, V. D., Rogero, S. O., Cruz, A. S., Vieira, D. P., Okazaki, K., Rogero, J. R. (2014). In vitro tests of resveratrol radiomodifying effect on rhabdomyosarcoma cells by comet assay. *Toxicology in Vitro*, 28(8), 1436-1442.

Mahale, N. B., Thakkar, P. D., Mali, R. G., Walunj, D. R., Chaudhari, S. R. (2012). Niosomes: Novel sustained release nonionic stable vesicular systems — An overview. *Advances in Colloid and Interface Science*, 183–184(0), 46-54.

Manconi, M., Sinico, C., Valenti, D., Lai, F., Fadda, A. M. (2006). Niosomes as carriers for tretinoin: III. A study into the in vitro cutaneous delivery of vesicle-incorporated tretinoin. *International Journal of Pharmaceutics*, 311(1–2), 11-19.

Manosroi, A., Wongtrakul, P., Manosroi, J., Sakai, H., Sugawara, F., Yuasa, M., Abe, M. (2003). Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. *Colloids and Surfaces B: Biointerfaces*, 30(1), 129-138.

Manosroi, J., Khositsuntiwong, N., Manosroi, W., Götz, F., Werner, R. G., Manosroi, A. (2013). Potent enhancement of transdermal absorption and stability of human tyrosinase plasmid (pAH7/Tyr) by Tat peptide and an entrapment in elastic cationic niosomes. *Drug Delivery*, 20(1), 10-18.

Marianecchi, C., Di Marzio, L., Rinaldi, F., Celia, C., Paolino, D., Alhaique, F., Esposito, S., Carafa, M. (2014). Niosomes from 80s to present: The state of the art. *Advances in Colloid and Interface Science*, 205(0), 187-206.

Matos, M., Gutiérrez, G., Coca, J., Pazos, C. (2014). Preparation of water-in-oil-in- water (W₁/O/W₂) double emulsions containing trans-resveratrol. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 442, 69-79.

Menasse, R., Hedwall P.R., Kraetz, J., Pericin, L., Riesterer, A., Sallmann, R. Ziel, Jaques, R. (1978) *Scand. Journal Rheumatology*. (Suppl.22) 5.

Moghassemi, S., Hadjizadeh, A. (2014). Nano-niosomes as nanoscale drug delivery systems: An illustrated review. *Journal of Controlled Release*, 185(0), 22-36.

Mugabe, C., Azghani, A. O., Omri, A. (2006). Preparation and characterization of dehydration–rehydration vesicles loaded with aminoglycoside and macrolide antibiotics. *International Journal of Pharmaceutics*, 307(2), 244-250.

Ndiaye, M., Kumar, R., Ahmad, N. (2011). Resveratrol in cancer management: where

are we and where we go from here? *Annals of the New York Academy of Sciences*, 1215(1), 144-149.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science*, 1(0), 1806-1815.

Nelson, G. (2002). Application of microencapsulation in textiles. *International Journal of Pharmaceutics*, 242(1–2), 55-62.

Olga, G., Styliani, C., Ioannis, R. G. (2015). Co-encapsulation of ferulic acid and gallic acid in hp-b-cyclodextrin. *Food Chemistry*, 185: 33-40.

Ortega, I., Wong, D. H., Villanueva, J. A., Cress, A. B., Sokalska, A., Stanley, S. D., Duleba, A. J. (2012). Effects of resveratrol on growth and function of rat ovarian granulosa cells. *Fertility and Sterility*, 98(6), 1563-1573.

Pando, D., Beltrán, M., Gerone, I., Matos, M., Pazos, C. (2015). Resveratrol entrapped niosomes as yoghurt additive. *Food Chemistry*, 170(0), 281-287.

Pando, D., Caddeo, C., Manconi, M., Fadda, A. M., Pazos, C. (2013b). Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol. *Journal of Pharmacy and Pharmacology*, 65(8), 1158-1167.

Pando, D., Gutiérrez, G., Coca, J., Pazos, C. (2013a). Preparation and characterization of niosomes containing resveratrol. *Journal of Food Engineering*, 117(2), 227-234.

Paolino, D., Cosco, D., Muzzalupo, R., Trapasso, E., Picci, N., Fresta, M. (2008). Innovative bola-surfactant niosomes as topical delivery systems of 5-fluorouracil for the treatment of skin cancer. *International Journal of Pharmaceutics*, 353(1–2), 233-242.

Saari, M., Vidgren, M. T., Koskinen, M. O., Turjanmaa, V. M. H., Nieminen, M. M. (1999). Pulmonary distribution and clearance of two beclomethasone liposome formulations in healthy volunteers. *International Journal of Pharmaceutics*, 181(1), 1-9.

Pinilla, C. M. B., Brandelli, A. (2016). Antimicrobial activity of nanoliposomes co-encapsulating nisin and garlic extract against Gram-positive and Gram-negative bacteria in milk. *Innovative Food Science and Emerging Technologies*, 36: 287-293.

Saini, N., Dang, P., Singh, D. (2011). Niosomes: a novel drug delivery system. *Drug Delivery*, 1, 4.

Shahidi, F. (2009). Nutraceuticals and functional foods: Whole versus processed foods. *Trends in Food Science & Technology*, 20(9), 376-387.

Shi, B., Fang, C., Pei, Y. (2006). Stealth PEG-PHDCA niosomes: Effects of chain length of PEG and particle size on niosomes surface properties, in vitro drug release, phagocytic uptake, in vivo pharmacokinetics and antitumor activity. *Journal of Pharmaceutical Sciences*, 95(9), 1873-1887.

Shi, G., Rao, L., Yu, H., Xiang, H., Yang, H., Ji, R. (2008). Stabilization and encapsulation of photosensitive resveratrol within yeast cell. *International Journal of Pharmaceutics*, 349(1–2), 83-93.

Shilpa, S., Srinivasan, B., Chauhan, M. (2011). Niosomes as vesicular carriers for delivery of proteins and biologicals. *International Journal of Drug Delivery*, 3(1).

- Shinde, T., Sun-Waterhouse, D., Brooks, J. (2014). Co-extrusion encapsulation of probiotic lactobacillus acidophilus alone or together with apple skin polyphenols: an aqueous and value-added delivery system using alginate. *Food and Bioprocess Technology*, 7: 1581-1596.
- Simard, P., Leroux, J.-C. (2009). pH-sensitive immunoliposomes specific to the CD33 cell surface antigen of leukemic cells. *International Journal of Pharmaceutics*, 381(2), 86-96.
- Soleas, G. J., Diamandis, E. P., Goldberg, D. M. (1997). Resveratrol: A molecule whose time has come? And gone? *Clinical Biochemistry*, 30(2), 91-113.
- Stratulat, I., et al. (2014). Enrichment of cheese with bioactive lipophilic compounds. *Journal of Functional Foods*, 6: 48-59.
- Stratulat, I., et al. (2015). Enrichment of cheese with vitamin D3 and vegetable omega-3. *Journal of Functional Foods*, 13: 300-307.
- Surh, Y.-J., Hurh, Y.-J., Kang, J.-Y., Lee, E., Kong, G., Lee, S. J. (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Letters*, 140(1), 1-10.
- Tavano, L., Muzzalupo, R., Picci, N., de Cindio, B. (2014). Co-encapsulation of antioxidants into niosomal carriers: Gastrointestinal release studies for nutraceutical applications. *Colloids and Surfaces B: Biointerfaces*, 114(0), 82-88.
- Terzano, C., Allegra, L., Alhaique, F., Marianecchi, C., Carafa, M. (2005). Non-phospholipid vesicles for pulmonary glucocorticoid delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, 59(1), 57-62.
- Tomé-Carneiro, J., Larrosa, M., Yáñez-Gascón, M. J., Dávalos, A., Gil-Zamorano, J., González, M., García-Almagro, F. J., Ruiz Ros, J. A., Tomás-Barberán, F. A., Espín, J. C., García-Conesa, M.-T. (2013). One-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease. *Pharmacological Research*, 72(0), 69-82.
- Uchegbu, I. F., Bouwstra, J. A., Florence, A. T. (1992). Large disk-shaped structures (discomes) in nonionic surfactant vesicle to micelle transitions. *The Journal of Physical Chemistry*, 96(25), 10548-10553.
- Uchegbu, I. F., Double, J. A., Kelland, L. R., Turton, J. A., Florence, A. T. (1996). The activity of doxorubicin niosomes against an ovarian cancer cell line and three in vivo mouse tumour models. *Journal of Drug Targeting*, 3(5), 399-409.
- Uchegbu, I. F., Vyas, S. P. (1998). Non-ionic surfactant based vesicles (niosomes) in drug delivery. *International Journal of Pharmaceutics*, 172(1-2), 33-70.
- Vian, M. A., Tomao, V., Gallet, S., Coulomb, P., Lacombe, J. (2005). Simple and rapid method for cis- and trans-resveratrol and piceid isomers determination in wine by high-performance liquid chromatography using Chromolith columns. *Journal of Chromatography A*, 1085(2), 224-229.
- Waddad, A. Y., Abbad, S., Yu, F., Munyendo, W. L., Wang, J., Lv, H., Zhou, J. (2013). Formulation, characterization and pharmacokinetics of Morin hydrate niosomes prepared

from various non-ionic surfactants. *International Journal of Pharmaceutics*, 456(2), 446-458.

Wagner, A., Vorauer-Uhl, K., Kreismayr, G., Katinger, H. (2002). The crossflow injection technique: an improvement of the ethanol injection method. *Journal of Liposome Research*, 12(3), 259-270.

Walde, P., Ichikawa, S. (2001). Enzymes inside lipid vesicles: preparation, reactivity and applications. *Biomolecular Engineering*, 18(4), 143-177.

Wang, B., Vongsivut, J., Adhikari, B., Barrow, C. J. (2015). Microencapsulation of tuna oil fortified with the multiple lipophilic ingredients vitamins A, D3, E, K2, curcumin and coenzyme Q10. *Journal of Functional Foods*, 19: 893-901.

Wang, M., Yuan, Y., Gao, Y., Ma, H.-M., Xu, H.-T., Zhang, X.-N., Pan, W.-S. (2012). Preparation and characterization of 5-fluorouracil pH-sensitive niosome and its tumor-targeted evaluation: in vitro and in vivo. *Drug Development and Industrial Pharmacy*, 38(9), 1134-1141.

Watson, D. S., Endsley, A. N., Huang, L. (2012). Design considerations for liposomal vaccines: Influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. *Vaccine*, 30(13), 2256-2272.

Wu, C.-F., Yang, J.-Y., Wang, F., Wang, X.-X. (2013). Resveratrol: botanical origin, pharmacological activity and applications. *Chinese Journal of Natural Medicines*, 11(1), 1-15.

Xiao, D., Davidson, P. M., Zhong, Q. (2011). Spray-dried zein capsules with coencapsulated nisin and thymol as antimicrobial delivery system for enhanced antilisterial properties. *Journal of Agricultural and Food Chemistry*, 59: 7393-7404.

Yu, H.-w., Wang, Y.-s., Li, Y., Shen, G.-L., Wu, H.-L., Yu, R.-Q. (2011). One Step Highly Sensitive Piezoelectric Agglutination Method for Cholera Toxin Detection Using GM1 Incorporated Liposome. *Procedia Environmental Sciences*, 8(0), 248-256.

Zarif, L., Gulik-Krzywicki, T., Riess, J. G., Pucci, B., Guedj, C., Pavia, A. A. (1994). Alkyl and perfluoroalkyl glycolipid-based supramolecular assemblies. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 84(1), 107-11.