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"Development of innovative formulations for topical administration of vitamin K1"

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General introduction

Vitamin K1

Vitamin K1 or phylloquinone (2-methyl-3-phenyl-1,4-naphthoquinone) (Figure 1) is a natural vitamin contained in vegetables, composed by an hydrophilic naphthoquinone group, and an hydrophobic tail (Gonnet M. *et al.,* 2010). It comes as a yellowish oil, somewhat viscous, soluble in fats and oils, in ethyl ether, benzene, chloroform, and insoluble in water. Vitamin K1 is the form of vitamin K mostly present in the diet. In the presence of light and atmospheric oxygen it may decompose, while it is heat resistant and does not undergo any processing up to 150 ° C.

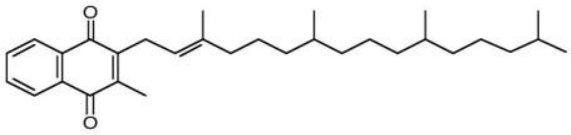

Figure 1. Structure of vitamin K1.

This molecule was discovered in 1929 by Henrik Dam who identified it as an anti-hemorrhagic factor (Da Silva *et al*., 2012); he studied the metabolism of the cholesterin in chickens maintained on a diet devoid of vitamin C, and he ascertained, after a certain time, the occurrence of a hemorrhagic syndrome (Dam H. *et al*., 1938). During his studies he was able to verify that, by administering to chickens in experiment some green leaves, this hemorrhagic syndrome disappeared completely. Subsequently, Dam and Schnheyder, concluded by stating that there is a new fat-soluble vitamin necessary to ensure the normal process of blood clotting in chicks; this vitamin was

called vitamin K or vitamin of the coagulation. Following McKee, Binkley, Mc Corquodale and Daisy isolated from the alfalfa plant a vitamin K, indicated with the number 1 (vitamin K1), to differentiate it from that obtained from the rotting fish meal (vitamin K2), (McKee R.W. *et al*., 1939). Almquist and Klose have explained that the active group of vitamin K (Figure 2), is the 2-methyl-1,4-naphthoquinone (Almquist H.J. *et al*., 1935).

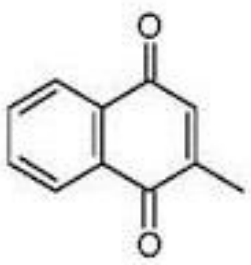

Figure 2. Active group of the vitamin K1.

Vitamin K is contained both in plants as in animals; in particular it is found in large quantities in some microorganisms. In plants it is contained mainly in the green leaves, and to a lesser extent also in fruits, tubers and seeds. The plant that contains this vitamin in excess is the alfalfa (a special variety of medical grass), then followed by the chestnut leaves, spinach, cabbage, cauliflower, nettle, pine and fir. Finally, many bacteria contain fairly large quantities of vitamin K, such as the *E. Coli*, *Bacillus subtilis*, *Staphylococcus Aureus* and many others; intestinal bacteria are also capable of producing vitamin K. Vitamin K has property antihemorrhagic; in fact, it acts as a co-enzyme of a carboxylase responsible for the carboxylation of glutamate residues. The carboxylation of these residues in prothrombin allows their complexation with calcium ions and the consequential activation of the same, followed by the coagulation cascade of events, which has as consequence the block of hemorrhagic phenomena (Gonnet M. *et al*., 2010). Several studies indicate that vitamin K is needed for the synthesis of osteocalcin, an important factor that contributes to the bone mineralization (De Orsi D. *et al*., 2008).

Effects of vitamin K1 on the skin

Topical application of vitamin K1 has been proven to be effective for the prevention of vascular events due to aging, for the suppression of pigmentation phenomena and for the resolution of bruising (Lou *et al*., 1999; Elson and Nacht, 1999). Phylloquinone was able to facilitate the removal of extravascular blood from the skin (Shah *et al*., 2002) thus determining its effectiveness in accelerating the resorption of bruises, as well as in countering any changes in the skin due to irradiation with laser beams (Lou *et al*., 1999; Elson and Nacht, 1999; Leu *et al*., 2010). Vitamin K1 has antioxidant activity and has a vasoprotective action probably due to its chemical structure similar to that of ubiquinone (Da Silva *et al*., 2012). However, because of its quinoid structure, the activity of the vitamin K1 could be greatly reduced by oxidation upon exposure to air or light (Gonnet M. *et al*., 2010).

Recently, it was found that creams containing vitamin K1 are very useful in the prevention of acneiform reactions affecting the skin following treatment with cetuximab in patients with metastatic colorectal cancer (Ocvirk *et al*., 2010). Cetuximab is a monoclonal antibody directed against the epidermal growth factor receptor (EGFR), which works by blocking the autophosphorylation and receptor-mediated signaling induced by endogenous ligands. EGFR is highly expressed in keratinocytes, sweat cells and sebaceous glands present in the epithelium of the hair follicles, and it appears to be important for the normal functions of the skin. It has been shown that the use of skin-EGFR inhibitors involves a series of reactions in the skin (about 80% of patients), among which the most common are acneiform reactions that appear frequently at the level of the head, neck, and trunk.

Other less frequent reactions are itching, dry skin, flaking and hypertrichosis (Segaeret *et al*., 2005; Van Custem *et al*., 2009). Recent studies have shown that the degree of acneiform reaction is greatly reduced in those regions of the body that are treated, prophylactically, with a cream containing vitamin K1. It has been shown that vitamin K1 activates EGFR, counteracting the inhibition induced by the use of Erbitux® (Tan E. *et al*., 2009; Li Th. *et al*., 2009). The action of vitamin K1 was associated with the up-regulation of phosphorylated EGFR, thereby reducing the adverse effects caused by the use of Erbitux (Tan E. *et al*., 2009; Li Th. *et al*., 2009). Topical application of

vitamin K1 was for this reason effective in ensuring a better quality of life of the patient, without dose reduction or discontinuation of the drug. (Ocvirk *et al*., 2010; Tomková *et al*., 2011).

Nanotechnologies in drug delivery

According with the US National Nanotechnology Intiative, Nanotechnologies can be defined as "all the materials and systems whose structures and components exhibit significant new physical, chemical and biological properties due to their nanoscale dimensions". Therefore, the term nanomedicine, means the union of two interdisciplinary fields, nanotechnology and medicine, representing a social and economic potential without precedent. For example, compared to conventional therapy in the treatment of cancer where the basic approach is to remove the diseased cells before the destruction of the healthy cells, the nanomedicine allows the use of sophisticated approaches to identify and kill or repair specific cells by the nanocarriers that can selectively directing diagnostic and /or therapeutic agents. An objective in this field is the design of molecular aggregates that have new dynamic and functional properties, desirable for applications in medicine. From a general point of view, the use of nanotechnology in medicine is aimed to overcome biological barriers, intended in the broad sense of the term. Thus, nanotechnology-based approaches can be designed to overcome biopharmaceutical issues at different levels, i.e. poor site- or cell-selectivity of drugs, drug instability in biological fluids, low cell uptake, low crossing of cellular barriers such as the blood brain barrier or the skin.

Nanotechnologies for topical use

Liposomes

The development of nanocarriers "specialized" in crossing the skin provided new possibilities for the development of formulations for topical use. Liposomes are self-assembling colloidal structures consisting of phospholipids, amphiphilic molecules that are arranged in a way that to create a phospholipid bilayer, concentrically arranged and

highly ordered. The constitution of the double layer is due to the nature of the liposomes; in fact, the amphiphilic molecules, if dispersed in an excess of water, will be oriented to form a double layer in which the polar and hydrophilic portions of the molecules will be in contact with the aqueous external environment (or internal), while the hydrophobic and apolar portions will remain intimately in contact with each other. Liposomes can be classified by the amount of lamellar vesicles (uni-, oligo- and multi-lamellar vesicles), size (small, intermediate or large) and the method of preparation (ie, reverse phase, evaporation of solvent or ethanol injection method). The unilamellar vesicles are formed from a single lipid membrane and generally have an average diameter between 50 and 250 nm. They contained a large aqueous compartment and are preferred for the encapsulation of hydrophilic molecules. The multi-lamellar vesicles, are constituted by more concentric lipid bilayers arranged concentrically (onion type) and have a mean diameter of between 1-5 μm. The structure of liposomes was developed in 1961 by Alec Bangham, (Bangham A, 1995; Gulati *et al.* 1998). By using *in vitro* models of plasma membranes, Alec Bangham showed the potential offered by these carriers in the pharmaceutical field. Liposomes were used since the early years in various fields such as diagnostics, immunomodulation, genetics, engineering and chemotherapy (Gregoriadis G.,1991). The biocompatibility of this lipid-based systems, thanks to the use of natural phospholipids, represented one of the major advantages of liposomes. Moreover, as delivery systems they are very versatile because they can encapsulate both hydrophilic and lipophilic molecules. The former are arranged in the lipid bilayer, while the latter can be entrapped within the aqueous core. Finally, also the amphiphilic molecules can be encapsulated; in this case the hydrophilic part being arranged in the aqueous medium and hydrophobic one placing in contact with the lipid constituents of the lamellae. The use of delivery systems such as liposomes have no restrictions of use for the different routes of administration and can easily be employed for topical, parenteral, oral, and pulmonary; for example, extremely lipophilic molecules can be administered without the requirement of surfactants, adjuvants or co-solvents that may be toxic. The physical-chemical nature of the drug and its therapeutic use strongly influence the choice of lipids to be used for the composition of the liposomal membrane.

The use of liposomes as carriers for topical therapy was introduced by

Gulasekharam and Mezei in 1980 (Mezei, 1980). They showed that the use of a lotion containing liposomes allowed to obtain levels of triamcinolone acetonide 4 or 5 times higher than those obtainable with conventional formulations containing the same drug concentrations. Similar results were also observed for progesterone and for econazole (Mezei, 1980). From these and other studies emerged that liposomes could be useful for the administration of several drugs to the skin (Ferreira *et al*., 2004; Puglia *et al*., 2004; Ramon *et al*., 2005; Kitagawa and Kasamaki, 2006). However, the lipid composition, the method of preparation and the thermodynamic characteristics of the bilayer of the liposomes, may considerably influence the drug penetration into the skin (Bouwstra and Honeywell-Nguyen, 2002; EI Maghraby *et al*., 2006).Other physico-chemical properties, such as size, charge and lamellarity can affect, even if to a lesser extent, the effectiveness of liposomes as carriers for administration of drugs to the skin. (Yu and Liao, 1996; Katahira *et al*., 1999; Ogiso *et al*., 2001; Liu *et al*., 2004; Manosroi *et al*, 2004; Choi and Maibach, 2005; Sinico *et al*., 2005). Mazei and Gulasekharm, (1980), found that intact and large vesicles, can penetrate the skin up to the dermis and failing to get into the capillaries, they formed a subcutaneous deposit system of drug. It was hard to believe that a large number of large lipid vesicles can penetrate the stratum corneum (El Maghraby *et al*., 2006). The explanation for this phenomenon could be provided by the presence of unilamellar, and not multilamellar, vesicles in the dermis initially applied on the skin. The authors proposed that liposomes can penetrate the epidermis losing their outer covering; alternatively, the enhanced drug penetration has been attributed to the surfactant activity of the liposome-forming lipids on the skin. In practice, they may act as enhancers penetration, loosening the lipid structure of the stratum corneum, promoting the alteration of the skin and, as consequence, the passage of the drugs (Kirjavainen *et al*., 1999a), although the greater interaction between the lipid of vesicles and those of the skin strongly depended on the lipid composition of the liposomes (Kirjavainen *et al*., 1996). This may explain the fact that the transport in the dermis of liposomes composed of lipids is much more pronounced than the transport of vesicles composed of phospholipids (Hofland *et al*., 1995; Korting *et al*., 1995; Zellmeret *et al*., 1995). Although liposomes have been successfully used to increase the accumulation of several drugs in the skin, there are also many cases of failure (Yu and Liao, 1996; Deo *et* al., 1997; Liu *et al*., 2004). This can probably be justified by the fact

that the liposomes did not cross the skin, but they remained confined to the outermost layer of the epidermis (Kirjavainen *et al*., 1996). Many research investigated the possibility to develop new types of carriers able to enhance the accumulation in the skin of encapsulated drugs or to promote the transdermal passage and therefore the absorption at the systemic level.

Transfersomes

Several studies have been aimed at improving the capacity of the nanocarriers to transport drugs into the deeper layers of the skin. Among these, ultraflexible liposomes, also named Transfersomes®,were designed by the research group of prof. Cevc (Cevc G. and Blume, 1992). Transfersomes are vesicles composed of phospholipids and a single chain surfactant such as sodium cholate or deoxycholate, Span 80 and Tween 80, which served as "edge activator", destabilizing the lipid bilayer and giving more flexibility to the carrier if compared with liposomes.(Cevc G. and Blume G., 2001; N. Bavarsad *et al*., 2012; Gillet A. *et al*., 2009). These nanocarriers being highly deformable are able to penetrate through the pores of the stratum corneum with a diameter ten times lower compared to their size; moreover, when applied on the skin under non-occlusive conditions, they could penetrate through the skin without altering their characteristics (Cevc G., 1996; El Maghraby G.M. *et al*., 2000a-b; Cevc G. and Blume G., 2001; Trotta M. *et al* 2004; Honeywell-Nguyen P.L *et al*.,2004). Because of the high elasticity of their membrane, these vesicles could penetrate inside the pores even when they were of large size, for example 200-300 nm (Vijaya R. *et al*., 2011; Mishra D. *et al*., 2007; Garg T. *et al*., 2008). Many studies have shown that these ultraflexible liposomes were able to promote the passage of drugs to the skin *in vitro* (El Maghraby *et al*., 1999, 2001a; Trotta *et al.*, 2002, 2004, Boinpally *et al*., 2003) and to penetrate the intact skin *in vivo*, carrying high amounts of drug (Cevc and Blume 2001, 2003, 2004) with an efficiency comparable to a subcutaneous injection (Cevc *et al*., 1995, 1998, Paul *et al*., 1995; Cevc, 2003).

Ethosomes

Ethosomes lipid-based vesicles were developed for the first time by the research group of Touitou (Touitou E., 1996; Touitou E *et al*.,1998; Touitou E *et al*., .2000). These vectors have proved very promising for administration of drugs with different nature on the skin, due to their properties to improve the passage of the encapsulated molecules through the barrier of the stratum corneum. (Touitou *et al*., 1997; Touitou *et al*., 2000a; Dayan e Touitou, 2000; Ainbinder e Touitou 2005; Paolino *et al*., 2005). Ethosomes were lipid carriers constituted by phospholipids, ethanol (concentration ranging from 20 to 45%) and water (Touitou *et al*., 2000a ; Elsayed, M.M. *et al*., 2007a). A number of studies have been performed to evaluate the effect of ethanol on the chemical and physical properties of vesicles. (Dayan e Touitou, 2000; Touitou *et al*., 2000a; Lopez-Pinto *et al*., 2005; Elsayed *et al*., 2007). Another important feature was the size of ethosomes lower than conventional liposomes (Dayan and Touitou, 2000). This has been attributed to the incorporation of high concentrations of ethanol which confers a net negative charge to the surface of liposomes, with consequent reduction in the vesicle size. Finally, the presence of ethanol also increases the solubility of several lipophilic active compound in the internal cavity of the vesicles. (Touitou E. *et al.,* 2000a; Lopez-Pinto *et al.*, 2005; Touitou E., 1996; Touitou E., *et al*., 1998; Ahad A. *et al*., 2013. Scognamiglio I. *et al*., 2013). In different studies, ethosomes were able to promote the passage of drugs into the skin both under occlusive or not occlusive conditions (Dayan e Touitou, 2000; Ainbinder e Touitou, 2005; Lopez-Pinto *et al*., 2005; Paolino *et al*., 2005; Elsayed *et al*., 2007). The effectiveness of ethanol in enhancing the permeation of molecules through the skin is well known; however, preliminary studies compared the permeation of drugs in hydroalcoholic solutions or delivered through these carriers have shown that the increase of diffusion through the skin obtained by the use of ethosomes is significantly higher in comparison with the use of an hydroethanolic solution alone. Therefore it was suggested a synergistic effect between the ethanol, the vesicles and the lipids of the skin and several mechanisms have been proposed to justify the efficient penetration and permeation of molecules encapsulated in ethosomes through the skin (Touitou *et al*., 2000a). For example, the multilamellar membrane of the stratum corneum, is densely packed and highly ordered at physiological temperature; ethanol could interact with the polar regions of the lipid

molecules, increasing the fluidity, and this involve an increase in membrane permeability; in addition, ethanol could confer a greater fluidity to the phospholipid bilayers which allowed to obtain vesicles with a more flexible structure in order to allow and facilitate their passage through the interstices of the stratum corneum (Berner B. *et al*., 1995). Furthermore, the release of the drugs into the deeper layers of the skin and their transdermal delivery could be the result of the fusion between ethosomes and the lipids of the skin (Elsayed *et a*l., 2006). In conclusion, due to the above characteristics mentioned above, ethosomes improve the permeation through the skin of both hydrophilic and lipophilic molecules. (Touitou E. *et al*., 2000a; Touitou E. *et al*., 2000b; Godin B. *et al*., 2003).

Nanoemulsions

In the last years, the nanoemulsions have been the subject of several studies that have evaluated the possibility of using these dispersed systems for the delivery of therapeutic agents and new hydrophobic chemical entities. With the term "nanoemulsion" referred to a nanodispersion of two immiscible liquids such as oil and water, which were thermodynamically stable for the use of surfactant molecules that arrange at the interface of the two phases. Nanoemulsions differ from emulsions for the size and shape of the dispersed particles in the continuous phase; in particular, nanoemulsions are characterized by particles of 20-300 nm. (Anton N. and Vandamme T.F, 2009; Bhavna Dhawan *et al*., 2014 Solans C., 1999). The small size of the dispersed particles make the nanoemulsions a thermodynamically stable system, while avoiding the occurrence of phenomena of alteration of the system, i.e. aggregation, flocculation and coalescence (Wen-Chien Lu *et al*., 2014).

In recent years, the micro and nano-emulsions gain a growing interest as a vehicle of active substances through the skin. The highest attention was paid to the oil-in-water nanoemulsions, which offered the possibility of dispersing the hydrophobic compounds in the oil phase, thus allowing to be dispersed in an aqueous medium and to increase their bioavailability *in vivo*. (Shafiq S. *et al*., 2007). Nanoemulsions can be designed with a high stability of the system for long time periods, low cost and easy

preparation. Moreover, it has been shown the ability of these systems to modify the penetration of a drug through the skin layers. (Shakeel F. *et al*., 2007; 2008; 2009). In fact, nanoemulsions could incorporate and carry the active substances through cell membranes (Huang Q. *et al*., 2010). It has been shown that the nanoemulsions facilitated the penetration of therapeutic agents into the skin and through it by increasing the concentration gradient of the drug in the various skin layers and altered the functionality of the skin barrier due to the presence of surfactants, which act as edge activators (Kreilgaard M., 2002a/b; A. Soottitantawat *et al*., 2005; Sateesh K. *et al*., 2007). Due to their advantages, the nanoemulsions were widely proposed for topical and transdermal delivery of antifungal and anti-inflammatory drugs, such as ketoprofen and meloxicam; however, it is important to emphasize that the size of the dispersed particles represented a crucial factor that could significantly influence the efficiency of the permeation of the active ingredients through the skin (Youenang Piemi M.P. *et al*., 1999; Yuan Y. *et al*., 2006; S. Hoeller *et al*., 2009; Sakeena M.H.F. *et al*., 2010; Zheng W.W. *et al*., 2010; Sonavane G. *et al*., 2008).

Aim of the work

The research activity has been focused on the study of new strategies for the dermal and transdermal administration of pharmacologically active molecules. In particular, the study was aimed to develop formulations based on nanocarriers for the administration of vitamin K1 (phylloquinone) in aerosol form on the skin. Recently, it was found that creams containing vitamin K1 was very useful in the prevention of acneiform reactions affecting the skin in patients receiving cetuximab for the treatment of metastatic colorectal cancer. (Ocvirk Janja *et al.,* 2010, Tomková *et al.,* 2011). Different semisolid formulations containing vitamin K1, namely Rencoval®, VigorSkinK1® and VigorSkinK1 Plus®, are already present on the market. The purpose of this study was therefore to develop alternative strategies for the administration of vitamin K1 on the skin, overcoming the drawbacks associated with the use, for more administration in a day, of fatty formulations. Therefore, innovative aqueous formulations to be administered on the skin by aerosol, thus avoiding the use of semisolid preparations, have been designed and developed in this work. To nebulize the developed formulations, a patented device (Eautè), portable and pocket with battery was used. In the development of vitamin K1-containing formulation alternative to the lipophilic formulations currently on the market, the possibility to increase the accumulation of vitamin in the epidermis and dermis was also taken into account. The formulative strategies used in this work start from previous published findings on lipid-based nanocarriers, such as liposomes, already used to administer different drugs on the skin. Then, the possibility to develop transfersomes and ethosomes to increase, compared to liposome-based formulations, the vitamin accumulation into the skin was investigated. Finally, the possibility to use lipid-free nanoemulsions, to propose formulative approaches less expensive for future commercial development, has been investigated. Thus, during all the experimental work, special attention was paid on the development of formulations suitable to be transferred to the industry (technological transfer). Thus, once designed the different formulations, their optimization has been aimed to combine high encapsulation of active compound, absent o low release of the

encapsulated molecule during storage, long shelf-life, as well as increased accumulation into the skin.

This PhD work has been carried out in collaboration with the Xenus s.r.l. in the respect of a confidential agreement between the PhD student, the University Tutor prof. Giuseppe De Rosa and the scientific responsible of the company dr. Michele Pitaro, some experimental details of this work have been omitted in this thesis.

Chapter 1

Development of a liposome-based formulation for nebulization of vitamin K1 on the skin

Abstract

Vitamin K1 (VK1) is a very lipophilic and photosensitive molecule contained in some vegetables. Recently, the use of VK1 on the skin has been proposed for different pharmaceutical or cosmeceutical applications. In this study, an innovative strategy for the administration of VK1 on the skin was proposed. In particular, to overcome the drawbacks associated with a VK1-containing fatty ointment available on the market, an aqueous formulation suitable to be administered by nebulization was developed. The use of liposomes encapsulating VK1 could represent a valid approach to overcome the issues due to the lipophilicity of VK1. Thus, different liposomal formulations, with different VK1 concentrations, were prepared and characterized in terms of size, zeta potential, VK1 encapsulation into liposomes, and stability of the formulations during storage. After a first phase of screening, the selected formulation was tested by a portable device for nebulization. No alteration of the vesicle characteristics following the liposome supplied through the nebulizer was found. Then, in a second phase, the possibility to add stabilizers, namely antimicrobic agents, antioxidants and a hydrogenated phospholipid, was evaluated.

Finally, permeation studies were carried out on pig-excised skin in Franz cells. In these tests, the VK1 permeation into the skin obtained with the newly developed formulations and with a marketed VK1-containing ointment were compared. An enhanced VK1 accumulation into the skin was found with all the liposomes formulations developed especially if administrated in the nebulized form. In conclusion, the newly developed formulations could be a valid alternative to the products available on the market today to administer VK1 on the skin. In particular, the use of liposomes could facilitate the multiple administrations *per* day by aerosol, but also increase, compared to a semi-solid preparation, the accumulation of VK1 into the epidermis and dermis.

Keywords: Vitamin K1, liposomes, liposome stability, liposome nebulization, Franz cells.

Introduction

Vitamin K1 (VK1) or 2-methyl-3-fitil-1,4-naphthoquinone (or phylloquinone) is a vitamin contained in different plants, especially as leaf vegetables and in smaller quantities also in fruits, tubers and seeds (Pace E., 1949). Although VK1 is well known as anti-hemorrhagic factor, other pharmacological activities and potential therapeutic or cosmeceutical uses are emerging in the last years. Due to its chemical structure similar to the ubiquinone, VK1 was found to act as an antioxidant agent (Da Silva A.L. *et al*., 2012). VK1 has been proposed to prevent vascular events due to aging, by suppressing the skin pigmentation and leading to resolution of bruising as well as countering changes in the skin after irradiation with laser beams (Lou W.W. *et al*., 1995; Elson M.L. *et al*., 1999; Leu S. *et al*., 2010). Finally, it has been shown that the topical application of VK1 is able to facilitate the removal of extravascular blood from the skin (Shah N.S. *et al*., 2012). In the last years, creams containing VK1 have been proposed to prevent side effects on the skin in patients with metastatic tumors of the colon and rectum, treated with cetuximab (Erbitux®), a monoclonal antibody directed against the epidermal growth factor receptor (EGFR), (Ocvirk J. *et al*., 2012). Indeed, the use of EGFR skin inhibitors involves a set of reactions in the skin (ca. 80% of patients), among which the most common are acneiform reactions that appear frequently at the level of the head, neck and trunk (Segaert S. *et al*., 2005; Van Cutsem E. *et al*., 2009). Recent studies showed that the degree of these acneiform reactions is strongly reduced in those regions of the body that are prophylactically treated with a cream containing VK1. This effect has been attributed to the capability of VK1 to activate the EGFR by up-regulation of EGFR phosphorylation, thus counteracting the cetuximab-induced inhibition of this receptor (Tan E. H. *et al*., 2009; Li T. H. *et al*., 2009). The topical application of VK1 is, for this reason, effective in ensuring a better quality of life of the patient, with no dose reduction or discontinuation of the therapy (Ocvirk J. *et al*., 2010; Tomková H. *et al*., 2011).

However, VK1 is yellow viscous oil, soluble in organic solvent and insoluble in water. The high lipophilicity of the VK1 requires fat ointments (e.g. Rencoval K1® ,VigorSkin K1®) making its multiple daily administrations on the skin poorly compliant,

especially in summer. In the light of these observations, the develop%ment of a formulation designed for a more practical and frequent administration of the VK1 on the skin, could be very useful allowing a prophylactic use of this vitamin with a higher patient compliance.

In last two decades, a growing number of studies have been dedicated to the use of nanotechnology-based approaches for topical administration of drugs. The use of colloidal systems, such as liposomes, can allow to increase the drug accumulation into the skin, depending on the type of drug as well as on the characteristics of the nanocarrier (Ferreira *et al*., 2004; Puglia C. *et al*., 2004; Ramón E *et al*., 2005; Kitagawa S. *et al*., 2006; Gokce EH *et al*., 2012; Hamishehkar H. *et al*., 2013).

Aim of the work

The aim of this study was to develop a new aqueous formulation containing VK1 suitable to be administered by aerosol (by a portable device) on the skin. In particular, we investigated if a formulation based on liposomes could be used to overcome the low water solubility of VK1. Thus, in a first step, different formulations were prepared and characterized to select liposomes encapsulating VK1 with the optimal technological characteristics, in terms of size, VK1 encapsulation and stability in different storage conditions. Then, the possibility to nebulize the liposomal suspension, without alteration of the vesicles, was evaluated. In a second step, the composition of the vesicle bilayer was optimized in order to obtain a more stable liposomes formulation. Finally, VK1 accumulation and permeation into and through the skin were investigated on Franz cells by using ear porcine skin

Materials and Methods

Materials

Vitamin K1 (VK1), α-tocopherol (αTOC) and iron thiocyanate ($FeSCN_3$) were purchased by Sigma (USA). HPLC grade methanol (CH_3OH) and acetonitrile (CH_3CN), analytical grade chloroform ($CHCl_3$) and ethanol (CH_3CH_2OH) were obtained from Carlo Erba Reagents (Italy). Benzalkonium chloride was provided by Farmalabor (Italy); soy phosphatidylcholine (SPC) and soy phosphatidylcholine hydrogenated were a kind gift by Lipoid GmbH (Switzerland).

Preparation of liposomes

The preparation of liposomes was performed by hydration of a lipid film followed by extrusion. Briefly, an organic solution ($CHCl_3/CH_3OH$ 2:1) containing SPC was dried in a round-bottom glass flask by a rotary evaporator (4010 Laborota digital, Heidolph, Schwabach, Germany) under a nitrogen atmosphere for about 20 min at 110 RPM and at a temperature of 30 °C. Only in the case of liposomes containing, hydrogenated soy phosphatidylcholine (LVB-HYDRO), the temperature of the rotary evaporator was set at 40°C. When present, VK1 and αTOC were added at the organic solution at different concentrations.

The obtained lipid films were then rehydrated, in the presence of glass beads, with a phosphate buffer solution (PBS) having pH of 7.4 or water of an aqueous solution 0.01% p/v of benzalkonium chloride. The resulting suspension was repeatedly passed through polycarbonate membranes (5 times for each membrane) of decreasing porosity (0.4, 0.2, 0.1 μm) by using a thermobarrel extruder (Northern Lipids Inc., Canada). Finally the liposomes were purified by molecular exclusion chromatography (SEC) with a Sephadex G-50-50, to remove the non-encapsulated VK1. For each preparation three batches were prepared.

Liposome size and zeta potential

Dimensional analysis was performed by photon correlation spectroscopy (PCS). For each sample, an aliquot of about 20µl was diluted in filtered water and analyzed by N5 (Beckman Coulter, USA). The average diameter and the size distribution of each formulation were determined. The results were expressed as liposome mean diameter (nm) and polydispersity index (PI).

The zeta potential (ZP) of liposomes was performed by the Zetasizer Nano Z (Malvern, UK). Briefly, an aliquot of each sample (20µl) was diluted in filtered water and analyzed. The results were calculated by the average of the measurements obtained from three batches of the same formulation.

Determination of lipids concentration

The concentration of lipids present into the liposome suspension after preparation was determined using the Stewart assay (Stewart J. C. M., 1959). Briefly, an aliquot of the liposome suspension was added to a two-phase system, consisting of an aqueous ammonium iron-thiocyanate solution (0.1N) and chloroform. The concentration of SPC was obtained by measure of the absorbance at 485 nm into the organic layer by UV-vis spectrophotometer (UV - VIS 1204 , SHIMADZU, Japan), by comparison with a calibration curve (R^2= 0.999) with standard SPC samples with a concentration ranging from 0.005 to 0.04 mg/ml.

VK1 encapsulation into the liposomes

The amount of VK1 encapsulated into the liposomes was determined by high performance liquid chromatography (HPLC). For the analysis, a Shimadzu HPLC system (Shimadzu) consisting on LC-10AD pump, a Rheodyne injection valve 7725i, equipped with a detector SPV-10A UV-vis (λ set at 333 nm) and a system controller (Shimadzu) SCL-10A VP connected to a computer, was also used. The analysis was performed on a Luna C8 column (250 x 4.6 mm, 5 um) (Phenomenex, USA) under

isocratic conditions and using CH_3OH as mobile phase at a flow of 1 ml/min. The acquisition of the chromatograms was carried out using the software Class VP Client/Server 7.2.1 (Shimadzu). The analysis in HPLC showed a chromatographic peak associated with the VK1 at a retention time of about 7 minutes. The amount of VK1 was calculated by comparison with a calibration curve (R^2= 0.999) with standard VK1 samples with a concentration ranging from 50 to 0.5 μg/ml. To determine the amount of VK1 encapsulated in liposomes, the suspension was diluted (1:100) with CH_3OH to allow the dissolution of the vesicles and the consequent release of VK1. The samples were then centrifuged for 30 min at 13,000 RPM (Mirko 20, Hettich, Germany) and then the supernatant was analyzed by HPLC. The results have been expressed as actual loading, calculated as μg of VK1 per mg of SPC. The results are the mean of measures made on three different batches.

Stability studies

Physical stability of liposomes was evaluated after different time frames on the formulations prepared at growing VK1 concentrations. Briefly, after preparation, each batch was stored at 4°C and at predetermined intervals, about 10 μl of the suspension was diluted in filtered distilled water and analyzed by PCS, as reported above.

The VK1 release from the liposome, at 4°C was determined on the formulations prepared at growing VK1 concentrations. Briefly, at predetermined intervals an aliquot, about 1 ml, of the suspension was purified by SEC. Then, the purified liposomes were analyzed in terms of VK1 content by HPLC, as reported above. The formulations of liposomes encapsulating VK1 were also tested in different storage conditions. In particular, liposomes were incubated at different temperatures, namely 4, 25 and 40°C, in refrigerant (Hotpoint, Ariston, Italy) or in a laboratory oven (STF-F52Lt, Falc Instrument, Italy). All samples were held under nitrogen atmosphere and protecting them from the light. In the case of sample stored at 25 °C, samples exposed to the sun light were also prepared. At predetermined intervals, the samples were analyzed in terms of physical appearance, odor, liposome size, VK1 content. Liposome size and VK1 content were determined by PCS and HPLC, respectively, as described above. For

each formulation, the results have been obtained as mean of three different batches (n = 3). The liposomes were also characterized before and after nebulization. To nebulize the liposome suspension, a portable nebulizer (Eauté) kindly provided by Xenus, was used. Briefly, about 1 ml of the liposome suspension, previously characterized for size and VK1 encapsulation, was loaded into the device and nebulized by collecting the aerosol in a 20 ml glass vial. The collected suspension was then analyzed in terms of vesicle size and VK1 encapsulation.

Skin penetration experiments

The penetration of VK1 in the skin and its transdermal delivery were assessed with porcine ear skin. The porcine ears were kindly provided by a local slaughterhouse (Vendor Carni, Italy). All the experiments were performed on frozen-thawed skin used within 6 months. Full-thickness skin was removed from the dorsal side of the freshly excised pig ear, stored at 20 °C and used within 6 months. On the day of the experiment, punches were cut out and hairs cut with scissors, as already reported by other authors (Gillet A., 2011). The outer skin surface (stratum corneum) and the inner region (dermis) were used for the experiments. After drying, the skin was cut into circles of 3 cm of diameter. For permeation experiments the skin was mounted in a Franz diffusion cells (Microglass Heim, Italy). Briefly, the porcine skin was mounted on the receptor compartment of a Franz diffusion cell assembly with the stratum corneum (SC) side facing upwards into the donor compartment. Seven milliliters of 3:7 (v/v) ethanol-pH 7.4 PBS was used as the receptor medium. A measured amount of liposomes containing VK1 was poured or nebulized into the donor compartment. Alternatively, a weighted amount of VigorSkinK1® cream was added into the donor compartment in contact with the excised skin. The concentration of all samples was adjusted to achieve same VK1 amount (1mg) in the donor compartment. The available diffusion area between compartments was 0.6 cm^2. The Franz cells were mounted on a H+P Variomag Labortechnik Telesystem (Germany) placed in a thermostatic bath Haake DC30 (Thermo Electron Corporation, Germany). The experiments were carried out at a stirring rate of 600 rpm and temperature of 37 °C. At predetermined time frames, 700 µl of the receptor phase were withdrawn and replaced with the same

amount of fresh medium. The amount of VK1 in the withdrawn samples was determined by HPLC. At the end of the experiments skin surface were thoroughly washed with distilled water to remove the excess formulation. Epidermis and dermis were then separated by heating and then placed in separate eppendorfs. VK1 accumulated into the skin was quantified as previously reported (Da Silva A., 2012; Lopes L.B. *et al*., 2007), with modifications. Briefly, the VK1 accumulated in epidermis or dermis was extracted with 1 ml of CH_3CN for 5 times by bath sonicator (Branson 3510) for 30 minutes. The CH_3CN phase was filtered using 0.45 μm membranes and the resulting filtrate analyzed by HPLC to determine the VK1 content. The amount of VK1 accumulated in the different layers of the skin was calculated as ratio between amount (ng) of VK1/ weight (mg) of epidermis or dermis.

Results

Characterization of the vesicles containing VK1

Liposomes were proposed for their capability to encapsulate hydrophobic molecules, such as VK1, and to form homogeneous colloidal dispersion in water. In the first phase of the work, three different liposomal formulations with increasing initial concentrations of VK1 were prepared and characterized in terms of size, VK1 encapsulation, physical stability and VK1 release during storage in different conditions. In Table 1, the developed formulations, as well as their characteristics in terms of mean diameter, PI, and ZP, are reported.

Formulation	Theoretical VK1 content (µg VK1/mg SPC)	Mean diameter (nm ± SD)	PI ± SD	ZP ± SD	Actual VK1 encapsulation (µg VK1/mg SPC ± SD)
LV	-	148.6 ± 6.2	0.104 ± 0.044	0.32 ± 0.40	-
LVA	2.5	131.1 ± 13.2	0.118 ± 0.031	-3.52 ± 2.10	3.4 ± 0.3
LVB	25	115.2 ± 7.2	0.135 ± 0.004	-3.43 ± 2.11	32.8 ± 8.1
LVC	125	147.0 ± 15.3	0.144 ± 0.018	-2.03 ± 0.46	154.0 ± 3.0

Table1: Characteristics of the formulations LV, LVA, LVB, LVC.

All the formulations shown a mean diameter < 150 nm. In particular, the formulation LV prepared without VK1 had a mean diameter of about 148.6 nm, that was higher compared to liposomes LVA and LVB (131.1 and 115.2 respectively). In the case of the formulation prepared with the higher concentration of VK1, liposomes diameter was similar to LV (147.0 nm). The differences in VK1 concentration encapsulated into liposomes did not influence size distribution (IP) of the vesicles that

was in all cases < 0.2 and specifically 0.104, 0.118, 0.135, 0.144 for LV, LVA, LVB, LVC, respectively. Only in the case of LV the value of ZP was neutral (0.32), while liposomes containing VK1 had a negative ZP that was not influenced by the VK1 concentration.

The amount of VK1 encapsulated into liposomes was determined by HPLC. As shown from the results, in all cases VK1 encapsulation was higher than the theoretical one. In particular, LVA had an actual loading of 3.4 µg VK1/mg SPC while for LVB and LVC VK1 encapsulation was 32.8 e 154.0 µg VK1/mg SPC. It is worthy of note that in all the three preparations, HPLC analysis of the liposome content showed only one chromatographic peak, attributed to the VK1, suggesting that the phylloquinone was not altered during the preparation (Figure 1).

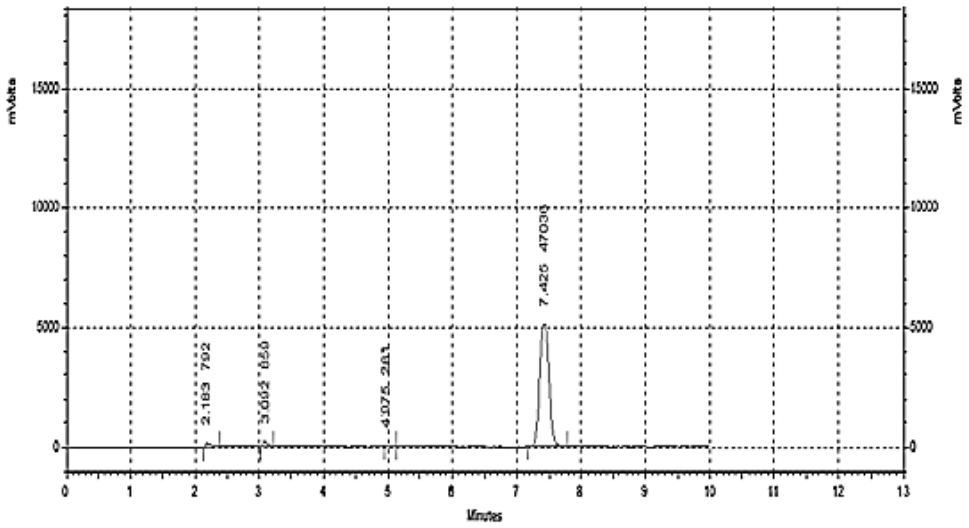

Figure 1 Exemple of chromatogram of VK1 extracted by LVB .

Note: The peak with the retention time of 7.3 minutes was attributed to native vitamin K1.

In the second step of the study, the stability of the developed formulations following storage at 4°C in absence of light was evaluated. The results are summarized in table 2.

Formulation	After 7 days			After 70 days			After 150 days		
	Mean diameter (nm ± SD)	**PI ± SD**	**Actual VK1 encapsulation (µg/mg SPC ± SD)**	**Mean diameter (nm ± SD)**	**PI ± SD**	**Actual VK1 encapsulation (µg/mg SPC ± SD)**	**Mean diameter (nm ± SD)**	**PI ± SD**	**Actual VK1 encapsulation (µg/mg SPC ± SD)**
LV	148.6±7.11	0.110± 0.04	-	148.5 ± 5.56	0.172 ± 0.03	-	151.2 ± 9.17	0.200 ± 0.01	-
LVA	137.2±15.38	0.118 ± 0.05	3.11 ± 1.22	140.8 ± 14.30	0.200 ± 0.05	3.06 ± 1.78	146.8 ± 0.23	0.246 ± 0.02	1.96 ± 0.182
LVB	118.4±3.05	0.134 ± 0.05	31.32 ± 3.14	122.2 ± 7.42	0.170 ± 0.02	22.55 ± 6.21	135.1 ± 4.53	0.190 ± 0.07	22.26 ± 11.08
LVC	148.9±17.61	0.157 ± 0.06	139.08 ± 35.44	UD	UD	UD	UD	UD	UD

Table 2: Stability of the formulations LV, LVA, LVB, LVC following storafe at 4 °C. Legend: UD: undetectable.

Blank liposomes did not showed significant alteration of the mean size and PI, following 150 days of storage at 4°C. Liposomes prepared with the lowest amount of VK1 (LVA) did not show significant change in mean size during storage, while VK1 loading was gradually reduced during storage. In particular, about 10% of the VK1 initially loaded into liposomes was lost during the first week of storage. In the following two months, VK1 loading of LVA did not significantly change; finally, a further and significant decrease of VK1 loading (to about 57% of the VK1 initial loading) was observed following 150 days of storage. In the case of LVB, about 5% of the VK1 was leaked from liposomes after the first 7 days of storage; a further and significant VK1 release from liposomes was also observed following 70 days of storage with a total VK1 release of about 30%; interestingly, the VK1 content of LVB did not significantly changed following further storage until 150 days. Moreover, the formulations LVA and LVB did not present any organoleptic alteration during storage. Different results were found for the formulation LVC, for which, agglomerates were visible in the suspension before 70 days of storage; moreover, a characteristic odor was reported for this formulation.

Thus, taking into account the obtained results, we considered that the formulation LVB had the best characteristics in terms of VK1 encapsulation and stability during storage to be used in the following step of the work.

Stability studies

The stability of the formulation LVB was investigated in different conditions of storage according to ICH guidelines (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use). Thus, a visual inspection to check the presence of agglomerate, colouring of the suspension, and organoleptic evaluation of the samples, were carried out at different time frames. In the different storage conditions, at the predetermined intervals of time, samples were characterized in terms of vesicle size and VK1 actual loading. In particular, any changes in terms of stability of the formulations stored at 25 °C, 40°C, and in the

presence of light, were studied. The results of the visual and organoleptic analysis are summarized in table 3.

Storage	After preparation			After 30 days			After 70 days		
	Aspect	**Odor**	**Colour**	**Aspect**	**Odor**	**Colour**	**Aspect**	**Odor**	**Colour**
4°C	Uniform	Absent	Milky	Uniform	Absent	Milky	Uniform	Absent	Milky
25°C	Uniform	Absent	Milky	Uniform	Slight odor	Milky	Uniform	Slight odor	Milky
40°C	Uniform	Absent	Milky	Uniform	Marked odor	Milky	Suspended material	Strong odor	Milky
Light at 25°C	Uniform	Absent	Milky	Uniform	Strong odor	Milky	Uniform	Strong odor	Milky

Table 3: Visual and organoleptic analysis of the formulation LVB stored in different conditions.

Liposome incubation at 40 ºC resulted in development of a pronounced characteristic odor after 30 days, while the presence of aggregation in the suspension was observed at longer time frames (i.e. 70 days). The development of a characteristic odor was significantly delayed and attenuated in samples stored at 25 °C (see table 3). Finally, in samples stored at 25 °C under the light, a marked odor was reported after the first 30 days, although not associated to the presence of aggregate. For all the samples, the storage under nitrogen atmosphere did not result in any influence of the suspension stability (data not shown). The same samples were also characterized in terms of liposome size and VK1 leakage during the storage (see table 4).

Storage conditions	After preparation			After 30 days			After 70 days		
	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)
4 °C	120.9 ± 8.7	0.107 ± 0.03	37.99 ± 8.54	120.4 ± 7.50	0.08 ± 0.03	34.55 ± 18.35	123.8 ± 0.10	0.133 ± 0.03	28.83 ± 8.69
25 °C				129.5 ± 6.15	0.085 ± 0.030	32.49 ± 9.00	134.1 ± 7.4	0.089 ± 0.04	43.73 ± 6.04
40 °C				126.3 ± 7.02	0.110 ± 0.049	41.24 ± 15.79	598.9 ± 18.71	0.579 ± 0.52	UD
Light at 25 °C				126.8 ± 3.45	0.109 ± 0.028	25.11 ± 5.11	132.6 ± 1.16	0.080± 0.73	20.13 ± 2.55

Table 4: Characteristics of the formulation LVB. Legend: UD: undetectable.

In agreement with the visual and organoleptic analysis, the samples stored at 40°C showed a marked increase of mean diameter and PI, suggesting the presence of aggregates into the suspension. For this reason, samples stored at 40°C did not undergo further characterization. In the case of liposomes stored in presence of light, only a moderate increase of the vesicle size was found, once more in agreement with previous observations (see table 3). It is worthy of note that, in sample exposed to the light, a reduction of the initially actual loading of VK1 (about 50%) was found. Moreover, HPLC analysis revealed additional chromatographic peaks (figure 2), with a retention time shorter, compared to that of native VK1. The additional compounds (with a main peak with retention time at 5.4) found in the HPLC analysis could be reasonably attributed to the transformation of the VK1 in its derivatives. This additional chromatographic peak was not found in the case of samples stored at 40°C in absence of light. On the other hand, in samples stored at high temperature, liposomes aggregation and enhanced VK1 release could occur.

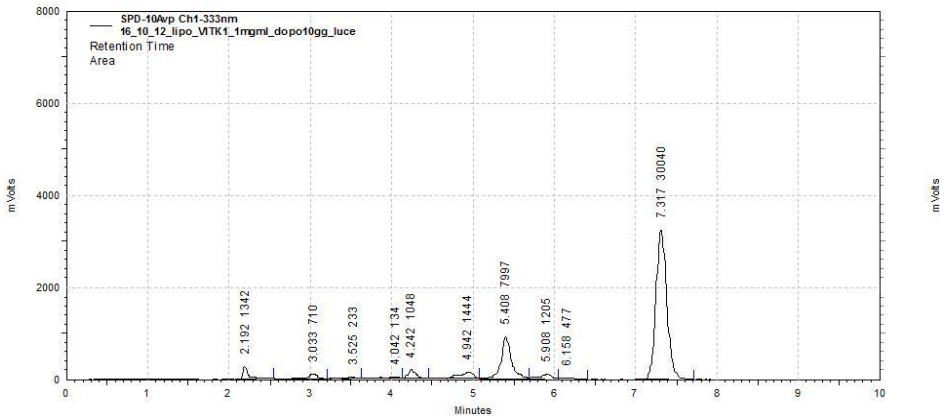

Figure 2 Chromatogram of sample extracted by LVB following storage under light.
Note: The peak with the retention time of 7.3 minutes was attributed to native vitamin K1.

In a second phase of the formulative study other two excipients were included in the formulation. Firstly, αTOC (10 µg/mg SPC) and benzalkonium chloride (0.01%)

were added in the organic solution containing the lipids and in the aqueous solution, respectively. αTOC was used to prevent the oxidation of lipids present in the bilayer, (Fukuzawa K, 2008). Benzalkonium chloride was added as antimicrobial preservative. This formulation, named LVB-TOC-BC1 was prepared with the same procedure used for LVB but an aqueous phase containing benzalkonium chloride was used as hydration phase of the lipid film. The results of the visual and organoleptic analysis of LVB-TOC-BC1 upon storage in different conditions, are summarized in Table 5. As shown, the formulation resulted altered after 30 days of storage at high temperature with presence of aggregates in the suspension, with development of a marked odor. Also samples stored at 25 °C were characterized by a slight odor, while the formulation appeared uniform.

Storage condition	**After preparation**			**After 30 days**		
	Aspect	**Odor**	**Colour**	**Aspect**	**Odor**	**Colour**
4°C	Uniform	Absent	Milky	Uniform	Absent	Milky
25°C	Uniform	Absent	Milky	Uniform	Slight odor	Milky
40°C	Uniform	Absent	Milky	Uniform	Marked odor	Milky
Light at 25°C	Uniform	Absent	Milky	Uniform	Slight odor	Milky

Table 5: Visual and organoleptic analysis of the formulation LVB-TOC-BC1 stored in different conditions.

In table 6, the liposomes characteristics in terms of size, VK1 encapsulation, and storage under different conditions are summarized. LVB-TOC-BC1 characteristics were very similar to that observed for LVB; in fact, liposomes had a mean diameter of about 113.9 nm after preparation and a IP < 0.2 that did not significantly changed during storage at 4 °C. The actual loading of VK1 was of about 40 μg VK1/mg SPC and was reduced of about 20% after 30 days. The results obtained from the organoleptic

analyses were confirmed by the results of liposomes characterization. In fact, after 30 days of storage at 40 °C, the mean diameter and the PI of the vesicles were high, about 549 nm and 0.54 respectively, and the measure of the VK1 was not possible confirming the alteration of the formulation. On the contrary, the alteration of the formulations stored at the temperature of 25 °C and in presence of light was not found in the liposome analyses.

In the following step of the study, the concentration of αTOC in the formulation was reduced in order to improve liposomes stability. Thus, liposomes with αTOC (2.5 μg/mg SPC) and benzalkonium chloride (0.01%) were prepared as described above.

Storage conditions	**After preparation**			**After 30 days**		
	Mean diameter (nm± SD)	PI± SD	Actual VK1 encapsulation (μg/mg SPC ± SD)	Mean diameter (nm± SD)	PI± SD	Actual VK1 encapsulation (μg/mg SPC ± SD)
4°C	139.1 ± 0.74	0.13 ± 0.00	40.72 ± 0.67	123.2 ± 1.3	0.13 ± 0.00	33.20 ± 1.3
25°C				135.4 ± 2.2	0.08 ± 0.00	33.41 ± 3.9
40°C				549.1 ± 48.2	0.54 ± 0.00	UD
Light at 25°C				133.3 ± 2.07	0.09 ± 0.00	25.11 ± 8.2

Table 6: Characteristics of the formulation LVB-TOC-BC1. Legend: UD: undetectable.

The characteristics of LVB-TOC-BC2 liposomes were not significantly different than LVB. In details, after preparation, liposomes have a mean diameter of about 110 nm with a PI of about 0.12. These characteristics did not significantly change following storage of the formulation at the temperature of 4°C (table 7). Moreover, LVB-TOC-BC2 liposomes had an actual loading similar to LVB, i.e. about 33 μg/mg SPC, that was reduced to about 25 μg/mg SPC after the first 7 days of storage at 4°C. Interestingly, further leakage of VK1 was not observed following storage at 4°C for more than 2 months.

Storage conditions	After preparation			After 7 days			After 70 days		
	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)
LVB-TOC-BC2	108.72 ± 9.56	0.122 ± 0.005	32.64 ± 6.28	111.13 ± 0.6	0.123 ±0.012	25.00 ± 2.4	114.48 ±1.41	0.124 ± 0.008	25.16 ± 0.99

Table 7: Characteristics of the formulation LVB-TOC-BC2.

Finally, the formulation LVB-TOC-BC2 was analysed in different conditions of temperature and light exposure. The results of the visual inspection and organoleptic analysis are reported in table 8, while in table 9 the liposomes characteristics in terms of size and VK1 encapsulation are summarized. LVB-TOC-BC2 showed a similar behavior compared to LVB, with presence of aggregation at 40°C and appearance of a characteristic and pronounced odor following liposome exposure to the light. Moreover, aggregation was not found in the suspension following liposome storage at 25 °C for 70 days.

Storage condition	**After preparation**			**After 70 days**		
	Aspect	**Odor**	**Colour**	**Aspect**	**Odor**	**Colour**
4°C	Uniform	Absent	Milky	Uniform	Absent	Milky
25°C	Uniform	Absent	Milky	Uniform	Absent	Milky
40°C	Uniform	Absent	Milky	Suspended material	Strong odor	Milky
Light at 25°C	Uniform	Absent	Milky	Uniform	Strong odor	Yellowish

Table 8: Visual and organoleptic analysis of the formulation LVB-TOC-BC2 stored in different conditions.

Storage conditions	**After preparation**			**After 70 days**		
	Mean diameter (nm ± SD)	**PI± SD**	**Actual VK1 encapsulation (µg/mg SPC±SD)**	**Mean diameter (nm ± SD)**	**PI± SD**	**Actual VK1 encapsulation (µg/mg SPC±SD)**
4°C	106.2 ± 1.6	0.091 ± 0.004	31.92 ± 6.5	114.1 ± 1.19	0.114 ± 0.007	37.99 ± 2.40
25°C				113.2 ± 1.45	0.111 ± 0.008	31.10 ± 5.32
40°C				588.3 ± 2.33	0.689 ± 0.03	UD
Light at 25°C				270.7 ± 30.62	1.053 ± 0.82	34.17 ± 6.37

Table 9: Characteristics of the formulation LVB-TOC-BC2. Legend: UD: undetectable.

Finally, the possibility to dispense the formulation LVB-TOC-BC2 in form of aerosol, for example with a portable device for nebulization, was investigated. Physical characteristics of liposomes were checked before and after the nebulization. The aerosol produced by nebulization of LVB-TOC-BC2 is shown in figure 3, while liposome characteristics before and after supply through the device are reported in table 10.

Figure 3. Nebulization of the formulation LVB-TOC-BC2 by a portable device.

Formulation	Mean diameter (nm ± SD) BN	PI ± SD BN	Mean diameter (nm ± SD) AN	PI± SD AN
LVB-TOC-BC2	117.6± 2.57	0.133 ± 0.003	120.8± 2.21	0.137± 0.002

BN: before nebulization; AN: after nebulization.

Table 10: Mean diameter and polidispersity index before and after nebulization of the formulation LVB-TOC-BC2.

Any alteration of liposome size and VK1 encapsulation was observed following nebulization, suggesting that the vesicles maintain their integrity when supplied in form of aerosol.

A third phase of the study was focused on the substitution of the soy phosphatidilcoline, used for the previous formulations, with the hydrogenated soy phosphatidilcoline, in order to obtain a formulation more stable during storage. In table 11, the characteristic of liposomes and the stability studies of LVB-HYDRO are summarized.

Storage consitions	After preparation				After 9 months			Aspect
	Theoretical VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm ± SD)	PI ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	
4 °C	50	135.2 ± 1.66	0.74 ± 0.037	80.61 ± 0.025	137.9 ± 0.707	0.193 ± 0.050	39.93 ± 0.007	Uniform
25 °C		-	-	-	194.9 ± 30.54	0.328 ± 0.164	30.48 ± 0.009	Uniform slight odor
40 °C		-	-	-	133.5 ± 27.15	0.190 ± 0.050	25.30 ± 0.006	Uniform slight odor

Table 11: characterization and stability studies of the formulation LVB-HYDRO.

As shown in table 11, the mean diameter of this liposomes formulation did not change after 9 months of storage of LVB-HYDRO at 4°C; in fact, the average diameter of liposomes after preparation was about 135.2 nm and remained unaffected after 9 months (137.9 nm). The value of the PI also remained < 0.2 during storage, while a VK1 loss of about 40 % after 9 months of storage of the formulation at 4°C was found. The results of the stability studies have evidenced that LVB-HYDRO was very stable compared to the previous developed formulations; indeed the organoleptic analyses did not shown any alteration in term of aspect of the formulation that remained uniform after 9 months of storage at 25 and 40 °C. It is worthy of note that a slight odor was reported in the case of the formulations stored at 40 and 25°C. The characterization analyses evidenced that after 9 months of storage of LVB-HYDRO at 25 °C the mean diameter and the PI slightly increased (194.9 nm and 0.3); after the same time frame, about the 60% of the initially encapsulated VK1 was observed (30.48 μg/mg SPC-HYDRO). The storage of LVB-HYDRO at higher temperature did not result in any significant alteration of the vesicles size; as reported, the mean diameter as well as the PI value liposomes remained unaffected (also more stable than in the case of liposomes stored at 25°C). On the other hand, the VK1 concentration strongly decreased from about 80.61 to 25.30 μg/mg SPC-HYDRO with a VK1 loss of about 69%.

Ex vivo experiments

The formulation LVB-TOC-BC2 was used for the *ex-vivo* studies using Franz cells. LVB-TOC-BC2 was used as such or nebulized through the medical device Nano Estté. VK1 permeation through the skin or accumulation into epidermis/dermis was compared with that obtained, in the same experimental conditions, with VigorSkink1®, a marketed cream containing VK1. Finally, to investigate if the excipients added in the formulation LVB, especially *α*TOC, influence VK1 delivery into and through the skin, LVB liposomes were also investigated in the skin penetration experiments. *Ex vivo* tests were carried out using Franz cells as describe above (Ferderber *et al.* J. Liposome Research 2009). The amount of VK1 accumulated into the epidermis and dermis are reported in figure 4A and B, respectively.

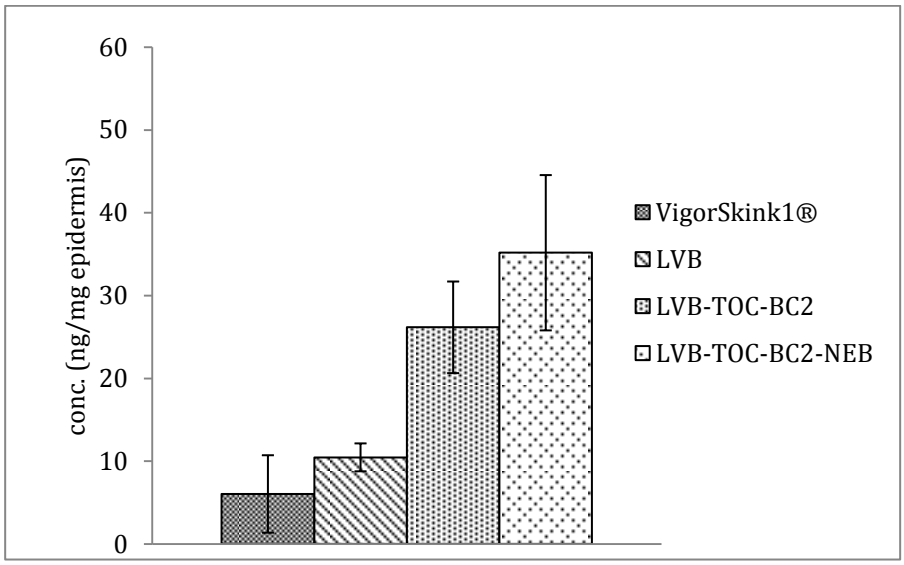

Figure 4A: Accumulation of VK1 in epidermis. Legend: VigorSkinK1®: LVB-TOC-BC2: liposomes containing VK1, αTOC and benzalkonium chloride, administrated as such in the donor compartment. LVB-TOC-BC2-NEB: liposomes containing VK1, αTOC and benzalkonium chloride, administrated in form of aerosol in the donor compartment.

In the case of skin samples treated with VigorSkink1®, VK1 accumulation in epidermis was very low (about 6.03 ± 4.68 ng/mg of epidermis). The amount of VK1 accumulated into the epidermis was significantly higher in the case of skin samples placed in contact with the liposomal-containing formulation (about 26.15 ± 5.53 ng/mg of epidermis for LVB-TOC-BC2), especially when the LVB-TOC-BC2 was applied on the skin by nebulization (35.16 ± 10.95 ng/mg of epidermis). The difference between liposome-based formulation and the marketed cream, was more evident in the case of the dermis, where any VK1 accumulation was found in the case of skin treated with VigorSkinK1® (Figure 4B).

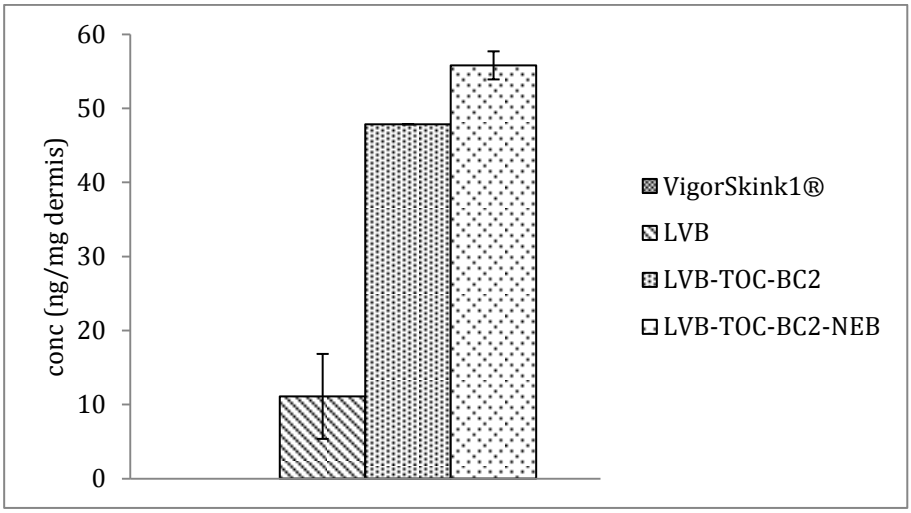

Figure 4B: Accumulation of VK1 in dermis. Legend: LVB-TOC-BC2: liposomes containing VK1, αTOC and benzalkonium chloride, administrated as such in the donor compartment. LVB-TOC-BC2-NEB: liposomes containing VK1, αTOC and benzalkonium chloride, administrated in form of aerosol in the donor compartment.

In fact, significant VK1 accumulation was found in dermis in the case of skin treated with both LVB-TOC-BC2 as such (47.8 ± 0.1 ng/mg of dermis) or nebulized (55.7 ± 1.89 ng/mg of dermis). In the case of LVB, the amount of VK1 in the epidermis was comparable to which observed with VigorSkinK1®; moreover, also LVB improved VK1 accumulation in the dermis even if less than LVB-TOC-BC2. Finally, for all samples, VK1 permeation through the skin was not observed.

Finally, in figure 5A and B the accumulation of VK1 found into the epidermis and dermis treated with LVB-HYDRO are reported. As shown in figure 5A, in the skin treated with LVB-HYDRO as such or in the aerosol form, the amount of VK1 accumulated into the epidermis was lower than in the case of LVB-TOC-BC2, namely about 22.44 and 27.58 ng VK1/mg of epidermis respectively for LVB-HYDRO and LVB-HYDRO-NEB. Also in the case of the accumulation of VK1 found in the dermis (figure 5B) the use of LVB-HYDRO as such or nebulized led to an accumulation of VK1 lower if compared to LVB-TOC-BC2. In particular, accumulated VK1 was about

32.60 and 46.58 ng VK1/mg of dermis respectively for LVB-HYDRO and LVB-HYDRO-NEB.

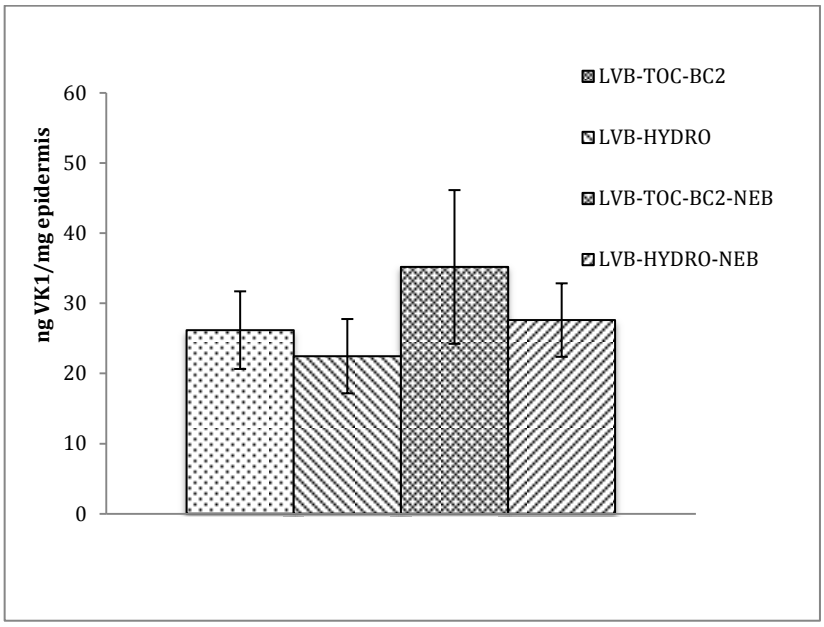

Figure 5A: Accumulation of VK1 in epidermis. Legend: LVB-TOC-BC2: liposomes containing VK1, αTOC and benzalkonium chloride, administrated as such in the donor compartment. LVB-TOC-BC2: liposomes containing VK1, αTOC and benzalkonium chloride, administrated in form of aerosol in the donor compartment. LVB-HYDRO : liposomes containing soy phosphatidilcoline hydrogenated and VK1, administrated as such in the donor compartment . LVB-HYDRO-NEB: liposomes containing soy phosphatidilcoline hydrogenated and VK1, administrated in form of aerosol in the donor compartment.

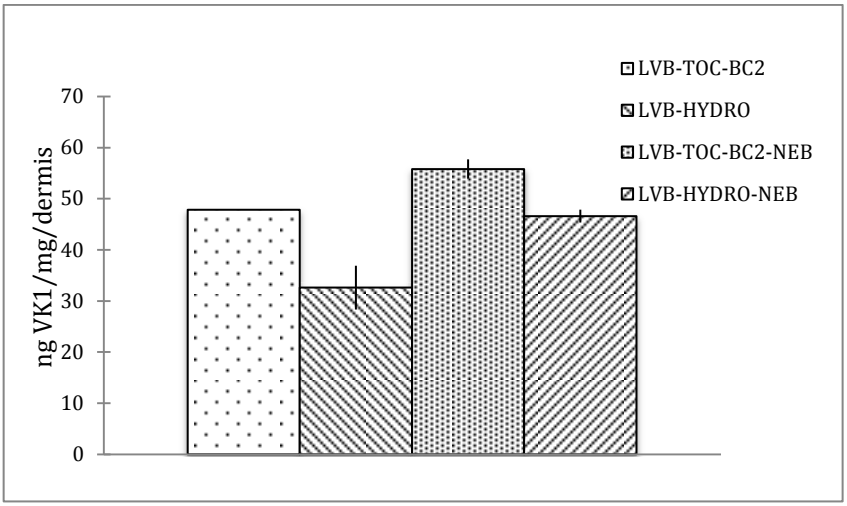

Figure 5B: Accumulation of VK1 into the dermis. Legend: LVB-TOC-BC2: liposomes containing VK1, αTOC and benzalkonium chloride, administrated as such in the donor compartment. LVB-TOC-BC2: liposomes containing VK1, αTOC and benzalkonium chloride, administrated in form of aerosol in the donor compartment. LVB-HYDRO : liposomes containing soy phosphatidilcoline hydrogenated and VK1, administrated as such in the donor compartment . LVB-HYDRO-NEB: liposomes containing soy phosphatidilcoline hydrogenated and VK1, administrated in form of aerosol in the donor compartment.

Discussion

The study was focused on the development of a new formulations for the topical application of VK1. In particular, the aim of the work was to design and develop an aqueous formulation, able to overcome the unpleasant feeling due to the use of a fat ointment on the skin, especially when used more times for a day. Moreover, to facilitate the administration of the VK1 on the skin, a formulation with a low viscosity, suitable to be nebulized in a portable device, was developed. In order to meet all these requirements, a formulation based on nanosized lipid vesicles, i.e. liposomes, was designed. Liposomes were proposed for their capability to encapsulate hydrophobic molecules, such as VK1, and to form homogeneous colloidal dispersion in water. To administrate the developed formulations in form of aerosol, a portable device nebulizer was used.

In the first phase of the work, three different liposomal formulations with increasing initial concentrations of VK1 were prepared and characterized in order to select the most suitable formulation in terms of diameter of vesicles, VK1 encapsulation and physical stability of the colloidal dispersion overtime and in stress condition. All the formulations were prepared with the same procedure, namely by the hydration of a lipid film followed by extrusion.. The developed formulations had a low mean diameter (in the range 150-100 nm), a narrow size distribution (<0.2), high VK1 encapsulation and good physical stability during storage. In addition, the presence of different concentrations of VK1 in the preparation did not significantly influence the mean diameter, as well as the size distribution with only a very slight influence on the liposome zeta potential.

The amount of VK1 encapsulated into liposomes was determined by HPLC analysis. For each formulation, the actual VK1 encapsulation was higher than the theoretical one. These finding could be explained with a lipid loss that was higher than the vitamin loss. This could be realistic if we hypothesized that the inclusion of VK1 into the lipid bilayer could result in the formation of vitamin-rich domains immiscible with the phospholipid bulk, according with other previously reported studies (Ortiz A.

et al., 1999). It is worthy of note that in all the three preparations, HPLC analysis of the liposome content showed only one chromatographic peak, attributed to the VK1, suggesting that the phylloquinone was not altered during the preparation. From the analyses carried out at predetermined time frame after storage of the formulation at 4 ºC, LVB showed the best characteristics in terms of VK1 encapsulation and stability while, LVC prepared with the higher concentration of VK1, was very instable as described above. Taking into account these results, it is possible to conclude that VK1 influence the physical stability of liposomes as well as the release of VK1 during the storage, depending on its concentration. It has been reported that VK1 can influence the properties of phospholipid bilayers by broadening and shifting the lipid transitions to lower temperature. Moreover, the formation of vitamin-rich domains, immiscible with the bulk phospholipids has been reported (Ortiz A. *et al*., 1999). These observations are in line with the results of this formulative study. In particular, the formation of these vitamin-rich domains could be favored when using high VK1 loading (formulation LVC). VK1 can be released from these domains and, consequently, cause an easier alteration of the bilayers of the vesicles, as testified by the development of unpleasant odor and the presence of aggregates, probably caused by a lipids alteration (e.g. peroxidation). Thus, we considered that the formulation LVB had the best characteristics in terms of VK1 encapsulation and stability during storage to be used in the following step of the work.

According with the ICH guidelines, stability studies with LVB were carried out; the aim of this part of the work was to evaluate how environmental factors, such as temperature and light exposure, can affect the product quality, thus establishing a shelf life for the drug product and recommending storage conditions. As described in the results, the formulation LVB was stored at different temperatures and some formulations were exposed to the light. From our results, we can hypothesize that, depending on the storage conditions, i.e. 40°C or light exposure, different mechanisms could be responsible of the sample alteration observed for LVB. In details, light exposure could result in the VK1 alteration, as evident by appearance of additional chromatographic peak. On the other hand, in samples stored at high temperature, liposome aggregation and enhanced VK1 release could occur. This physical instability of vesicles should be due to the presence of VK1 that could affect the bilayer

characteristics, as hypothesized above. In fact, blank liposomes were found to be stable, at the same temperature and for the same time frames. Thus, VK1 encapsulation into the liposomes should affect physical characteristics of the bilayer, making vesicles more unstable and enhancing VK1 release, following incubation at high temperature. Finally, it is worthy of note that a higher VK1 actual loading can be observed after storage of liposomes, especially at 40°C. Changes in the SPC and VK1 concentrations during storage in a liposome suspension should be due to different reasons. Actually, in the case of the VK1, as hypothesized above, a reduction of the concentration into the liposomes could be due to its release from the vesicle or to chemical alteration of the vitamin. In the case of SPC, a reduced content into the liposome suspension should be mainly ascribed to lipid hydrolysis. It is well known that high temperature favor lipid degradation, that can be minimized at low temperature (Pietzyk B. *et al*., 2000; Grit M. *et al*., 1993). In agreement with this, we found that, in the case of liposomes stored at high temperature, a decrease of the lipid concentration was found in samples stored at high temperature, with a consequent increased of the vitamin/lipid ratio (actual loading).

Subsequently, we tried to optimize the stability of LVB; in particular, αTOC was used to prevent the oxidation of lipids present in the bilayer (Fukuzawa *et al*., 2008) while, benzalkonium chloride was added as antimicrobial preservative. Two formulations named LVB-TOC-BC1 and LVB-TOC-BC2 with two different amounts of αTOC were developed and characterized as for LVB. The stability studies have evidenced that LVB-TOC-BC1 was unstable after 30 days of storage at high temperature and in samples stored at 25 °C some signs of alteration compared after only 30 days of storage. On the contrary, in the case of LVB-TOC-BC2, liposomes showed a similar behavior compared to LVB, with presence of aggregation at 40°C and appearance of a characteristic and pronounced odor following liposome exposure to the light. Moreover, aggregation was not found in the suspension following liposome storage at 25 °C for 70 days, suggesting that phospholipid oxidation could play a role in the suspension instability found at high temperature. Tests conducted with the formulation before and after nebulization demonstrated that the passage through the device did not affect the integrity of the vesicles confirming that LVB-TOC-BC2 was able to be administrated in form of aerosol.

In the third part of the study the natural lipid, soy phosphatidilcoline was replaced with a semisynthetic lipid, the hydrogened soy phosphatidilcoline. While SPC used in the previous formulations had double bonds on the hydrophobic tail, hydrogenated phosphatidilcoline is characterized by fully saturated fatty acids. The introduction of sully saturated lipids in the formulation, was made to prevent liposome alteration, attributed to the oxidative processes of the phospholipids. The characterization of LVB-HYDRO confirmed this hypothesis, showing that the formulation remained stable until 6 months of storage at high temperatures.

Several studies in literature have already described the capability of liposomes to improve the accumulation of different molecules into the skin (Ortiz A. *et al.,* 1999). Different advantages concerning the use of liposomes for the drug administration on the skin, have been described (Pierre M.B. *et al.,* 2011). Enhanced VK1 accumulation into the skin has already been described by using a nanodispersed monoolein-based formulation (Lopes L.B. *et al*., 2007) From a general point of view, liposomes can improve local drug concentration, by interacting with similar lipids of the skin (Pierre M.B. *et al*., 2011). The role of phospholipids as penetration enhancers, especially when containing unsaturated fatty acids, is well known. Phospholipids, such as SPC, can be easily incorporated within the viable cells via intercellular lipids of the outer layer of the skin; this can disrupt the lamellar structure stratum corneum with consequent increased fluidity and altered permeability (Ogiso T., 1995; Yokomizo Y., 1996). Thus, the use of SPC in dermatological formulations can results in enhanced diffusion of drug, especially lipophilic drugs, in the lipid domains and increased transport through the skin. While the use of a hydrophilic excipient, such as benzalkonium chloride, is expected to have no effect on the VK1 delivery into the skin (data not shown), the use of αTOC in the formulation dramatically increased the VK1 accumulation into epidermis and dermis. αTOC into the liposomes has already been associated to SPC as penetration enhancer (Lee W.C., 2010). αTOC, when encapsulated into the liposomes, have been found not only in the stratum corneum, but also in the underlying skin (Baschong W., 2001). Once into the skin, αTOC could synergistically act with SPC by altering the structure of the stratum corneum, thus enhancing the VK1 diffusion and accumulation into the epidermis and dermis. Interestingly, when including αTOC into a liposome formulation, an increased biocompatibility of liposomes on human fibroblast

has also been reported (Berrocal M.C., 2000). Finally, it is worthy of note that VK1, if systemically release at high concentrations, could alter the synthesis of coagulation factors. In this study, VK1 permeation through the skin was not found, suggesting a negligible systemic effect with this formulation.

Conclusions

In this study the possibility to develop a formulation for the administration of a highly lipophilic VK1 on the skin by nebulization was investigated. Liposomes can be valid tool to overcome the lipophilicity of VK1, forming a stable aqueous dispersion that can be administered on the skin in form of aerosol by means of a portable nebulizer without alteration of vesicle characteristics. Liposomes containing VK1 are stable if stored at 4°C in absence of light. Finally, the use of liposomes, as such or administered by nebulization, resulted in an enhanced VK1 accumulation into epidermis and dermis, when compared with a marketed fat VK1-containing cream. Our results suggest that the newly developed formulations can be considered a valid alternative to fat ointments for the administration of VK1 on the skin. This strategy for VK1 administration can be especially useful in the prevention of acneiform reactions against the skin following treatment with cetuximab in patients with metastatic tumors of the colon and rectum.

Chapter 2

Nanovectors to enhance the accumulation of vitamin K1 into the skin.

Abstract

In this part of the study lipid-based nanocarriers, namely liposomes, transfersomes and ethosomes for topical administration of VK1 were designed and developed. In the design of the formulations, special attention was paid to selection of preparation method very cheap and easy to perform. For each formulation, the possibility to administer these formulation in the aerosol form was also evaluated . The formulations developed were stable in different storage conditions for about 60 days and the deformability of transfersomes and ethosomes bilayers were strongly higher compared to liposomes one. In addition, the nebulization of transfersomes and ethosomes increased the accumulation of VK1 into the deeper skin layers without affecting the organization of the main components of the skin. and the different advantages offered by these kind of nanocarriers were discussed.

Keywords: Vitamin K1, transfersomes, ethosomes, Franz Cells, nebulization.

Introduction

The skin is an interesting route of administration of drugs, alternative to intravenous. In fact, it would appear to offer significant advantages such as, more stable plasma drug levels, exceeding the first-pass metabolism, and finally, a good compliance (Williams, 2003). However, the skin represent a natural barrier that hamper the permeation of many drugs (El Maghraby *et al*., 2001b). In the last two decades, a growing number of studies have been dedicated to the use of nanotechnology-based approaches to promote the accumulation of drugs into the skin or, sometimes, the transdermal delivery of drugs. The success of this approach is demonstrated by the number of products based on nanotechnology for topical administration that are today on the market or in clinical trials (Pierre M.B., 2011; Elsayed, M.M. *et al*., 2007 a e b). Although some studies suggested the use of liposomes for transdermal delivery of certain drugs (Yu and Liao, 1996; Deo *et al*., 1997; Liu *et al*., 2004), their capability to promote drug permeation through the skin or the accumulation in the deepest layers of the skin has been questioned, suggesting that liposome remain confined in the outermost layer of the skin. Thus, new generations of carriers for dermal administration of drugs have been proposed. Among these, a new type of lipid vesicles named ultraflexible liposomes or Transfersome® were firstly described by the research group of professor Gregor Cevc (Blume and Cevc, 1992). Transfersomes, characterized by a high degree of deformability due to the presence of an edge activator, can promote penetrate the intact skin carrying high amounts of actives in its deeper layers (El Maghraby *et al*., 1999, 2001a; Trotta *et al*., 2002, 2004; Boinpally *et al*., 2003; Cevc and Blume 2001, 2003, 2004). A further innovation in this field is certainly represented by the ethosomes, developed by Touitou and collaborators (US Patent N° 5,540,934, 1996). These vesicle, have been described as "soft" vesicles, composed of lipids, water and high concentration of ethanol. They are characterized by a high efficiency transdermal delivery of drugs with different nature. The skin permeation enhancement effect of ethosomes could be attributed to a different processes and, in particular, to the dual fluidizing effect of ethanol firstly on the phospholipids vesicles as well as on the lipids of the stratum corneum; the consequence is an easy penetration of these malleable

vesicles between the disturbed stratum corneum lipid bilayers (Touitou *et al*., 1997, 2000a; Dayan and Touitou, 2000; Ainbinder and Touitou 2005; Pauline *et al*., 2005).

Aim of the work

The aim of the work described in this chapter was to study formulations able to optimize the accumulation of VK1 in epidermis and dermis. In Chapter 1, a new aqueous formulation based on liposomes encapsulating VK1 (LVB-TOC-BC2) was designed. This study demonstrated that LVB-TOC-BC2 was able to improve VK1 accumulation into the skin in comparison with the marketed product Vigorskin K1®. Moreover, the nebulization of this formulation enhanced the accumulation of the active compound into the epidermis and the dermis. The goal of this part of the work was to develop formulations based on lipid-carriers, encapsulating VK1 such as transfersomes® (Trans) and ethosomes (Etho) to promote and improve the accumulation and the penetration of VK1 into/trough the skin. All the formulations were characterized in terms of size, VK1 encapsulation, physical stability and VK1 release during storage in different conditions. The deformability properties of the vesicles were also tested. In line with the scope of the previous chapter, we verified if the developed formulations were suitable to be administered on the skin by nebulization. Finally, the ability of different vesicles carriers to allow VK1 accumulation and permeation of VK1 into and through the skin was investigated in Franz diffusion cells, using ear porcine skin following incubation with different nanocarriers.

Materials and methods

Materials

VK1, α-tocopherol (αTOC), sodium cholate (SC), Sephadex G-50-50 and iron thiocyanate ($FeSCN_3$) were purchased from Sigma-Aldrich (St Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade methanol (CH_3OH) and acetonitrile (CH_3CN), analytical grade chloroform ($CHCl_3$) and ethanol (CH_3CH_2OH or EtOH) were obtained from Carlo Erba Reagents (Cornaredo, Italy). Soy phosphatidylcholine (SPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein-ammonium salt) (PE-CF) were kindly gifted by Lipoid GmbH (Steinhausen, Switzerland).

Preparation of VK1-encapsulating Liposomes, Transfersomes and Ethosomes.

Liposomes (Lipo) were obtained as follows according to the ethanol injection method (Batzri *et al*. 2003). SPC (10 mg/ml), VK1 (1 mg/ml) and αTOC (0.1 mg/ml) were dissolved in ethanol. The organic solution was slowly injected into a 10 ml vial containing distilled and 0.22 μm filtered water until an EtOH/H_2O ratio of 1:6 v/v; the obtained suspension was stirred at 1400 rpm for 15 minutes. To facilitate the removal of ethanol, the organic solvent was evaporated by rotary evaporation under reduced pressure at 37 °C. Finally, the liposomes were purified by molecular exclusion chromatography with a Sephadex G-50-50 (Sigma-Aldrich) to remove the non-encapsulated VK1. All the steps of the preparation were carried out avoiding exposure of VK1 to the light. The formulation was prepared in triplicate.

Transfersomes (Trans) were prepared by the thin lipid film hydration method as previously reported (Scognamiglio et al., 2012). Briefly, SPC (50 mg/ml) and SC (10 mg/ml), were dissolved in a mixture of chloroform/methanol (2:1 v/v) while VK1 (1 mg/ml) and αTOC (0.1 mg/ml) were dissolved in $CHCl_3$ .The organic solution was dried in a round-bottom glass flask by a rotary evaporator (4010 Laborota digital; Heidolph,

Schwabach, Germany) under a nitrogen atmosphere for about 20 minutes at 110 rpm and at a temperature of 40°C. The resulting film was then hydrated with distilled and filtered water by agitation on vortex in presence of glass beads. The resulting suspension was then extruded using a thermobarrel extruder system (Northern Lipids Inc., Burnaby, BC, Canada) passing the suspension under nitrogen through polycarbonate membrane (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK) with decreasing pore size (from 0.4 to 0.1 μm). The unencapsulated VK1 was removed by molecular exclusion chromatography by passing the nanocarrier suspension through Sephadex G-50-50 column. All the steps of the preparation were carried out avoiding exposure of VK1 to the light. The formulation was prepared in triplicate.

Ethosomes formulations were prepared according to the method described by Touitou *et al*. (2000), with modifications. Briefly, SPC (10 mg/ml), αTOC (0,1 mg/ml) and VK1 (1 mg/ml) were dissolved in ethanol. The resulting organic solution of lipids, αTOC and VK1 was added slowly and in constant mixing at 700 rpm to a 10 ml vial containing distilled and filtered water. Mixing was continued for an additional 5 minutes. The concentration of the organic solvent in the final formulation was 30%. This formulation was named Etho1. Alternatively, before purification, the suspension was extruded using a thermobarrel extruder system, passing the suspension under nitrogen through polycarbonate membrane with decreasing pore size (from 0.4 to 0.1 μm). For all the ethosomes-based formulations, the unencapsulated VK1 was removed by molecular exclusion chromatography by passing the nanocarrier suspension through Sephadex G-50-50 column. All the steps of the preparations were carried out avoiding exposure of VK1 to the light. Each formulation was prepared in triplicate.

Mean diameter and size distribution of the nanocarriers

Dimensional analysis was performed by photon correlation spectroscopy (PCS). For each sample, an aliquot of approximately 20 μL was diluted in filtered water and analyzed by PCS (N5; Beckman Coulter, Brea, CA, USA). The average diameter and the size distribution of each formulation were determined. The results were expressed as liposome mean diameter in nanometers and polydispersity index (PI).

Zeta potential of the nanocarriers

The zeta potential (ZP) of liposomes was performed by the ZetasizerNano Z (Malvern Instruments, Worcestershire, UK). Briefly, an aliquot of each sample (20 μL) was diluted in filtered water and analyzed. The results were calculated by the average of the measurements obtained from three batches of the same formulation.

Determination of lipid concentration

The concentration of lipids present in the suspensions after preparation was determined using the Stewart assay (Stewart *et al*., 1959). Briefly, an aliquot of the suspension was added to a two-phase system consisting of an aqueous ammonium ferrothiocyanate solution or iron thiocyanate solution (0.1 N) and $CHCl_3$. The concentration of SPC was obtained by measuring the absorbance at 485 nm into the organic layer with an ultraviolet–visible spectrophotometer (UV VIS 1204; Shimadzu Corporation, Kyoto, Japan). Quantification of SPC was carried out by means of a calibration curve ($r2$=0.999) with standard SPC samples at concentrations ranging from 0.005–0.04 mg/mL.

VK1 encapsulation into the nanocarriers

The amount of VK1 entrapped in the nanocarriers was determined by HPLC. For the analysis, a Shimadzu HPLC system, consisting of an LC-10AD pump and a Rheodyne injection valve 7725i, equipped with an SPV-10A ultraviolet–visible detector (λ set at 333 nm), and a SCL-10AVP system controller (Shimadzu) connected to a computer. The analysis was performed on a Luna C8 column (250×4.6 mm, 5 mm; Phenomenex, Torrance, CA, USA) under isocratic conditions and using CH_3OH as mobile phase at a flow of 1 mL/minute. The acquisition of the chromatograms was carried out using Class VP Client/Server software (v 7.2.1; Shimadzu Scientific

Instruments, Columbia, MD, USA). The analysis in HPLC showed a chromatographic peak associated with the VK1 at a retention time of about 7 minutes. The amount of VK1 was calculated by means of a calibration curve (r^2=0.999) with standard VK1 samples at concentrations ranging from 50–0.5 μg/mL. To determine the amount of VK1 encapsulated in liposomes, the suspension was diluted (1:100) with CH_3OH to allow the dissolution of the vesicles and the consequent release of VK1. The samples were then centrifuged for 30 minutes at 13,000 rpm (MIKRO 20; Hettich, Tuttlingen, Germany) and then the supernatant was analyzed by HPLC. The results are expressed as actual loading, calculated as μg of VK1 per mg of SPC. The results are the mean of measures made on three different batches.

Deformation index of the nanocarriers

The deformability of lipids vesicles was determinated by the extrusion method. Briefly , liposomes, transfersomes and ethosomes were extruded trough a polycarbonate membrane of 50 nm at the pressure of 1 MPa using a thermobarrel extruder system (Northern Lipids Inc., Burnaby, BC, Canada). The deformability of the nanocarriers was expressed as deformation index (DI) trough the equation 1:

$$DI = J\,(d_0/p)^k\,(1/|d_1 - d_0|)$$

where J is the quantity of suspension recovered after extrusion, d_0 and d_1 are the mean diameters of the nanocarriers before and after extrusion, k is an amplification factor and p is the pore size of the extruder membrane (Mura *et al*., 2009).

Stability studies

Physical stability of the nanocarriers was evaluated after different time frames on the formulations prepared using increasing VK1 concentrations. Briefly, after preparation, each batch was stored at 4°C and, at predetermined intervals, approximately 10 μL of the suspension was diluted in filtered distilled water and analyzed by PCS, as reported above.

The VK1 release from the nanocarriers at 4°C was determined in the different VK1 formulations. Briefly, at predetermined intervals, an aliquot of approximately 1 mL of the suspension was purified by molecular exclusion chromatography. Then, the purified nanocarriers were analyzed in terms of VK1 content by HPLC, as reported previously. The formulations encapsulating VK1 were also tested in different storage conditions. In particular, nanocarriers were incubated at different temperatures, namely 4°C, 25°C, and 40°C, in a refrigerator (Indesit Company SpA, Fabriano, Italy) or in a laboratory oven (STF-F52Lt; Falc Instruments, Treviglio, Italy). During this test, all samples were protected from light. At predetermined intervals, the samples were analyzed in terms of physical appearance, odor, vesicles size, and VK1 content. Vesicles size and VK1 content were determined by PCS and HPLC, respectively, as described previously. For each formulation, the results were obtained as the means of three different batches (n=3). The carriers were also characterized before and after nebulization. To nebulize the suspensions, a portable nebulizer (Eauté, Xenus, Rome, Italy), kindly provided by Xenus (Rome, Italy), was used. Briefly, approximately 1 mL of the nanocarrier suspension, previously characterized for size and VK1 encapsulation, was loaded into the device and nebulized by collecting the aerosol in a 20 mL glass vial. The collected suspension was then analyzed in terms of size and VK1 encapsulation. The results are the mean of measures made on three different batches.

Skin penetration experiments

The penetration of VK1 into the skin and its transdermal delivery were assessed using porcine ear skin. The porcine ears were kindly provided by a local slaughterhouse

(Vendor Carni, Montefredane, Italy). Full-thickness skin was removed from the dorsal side of the freshly excised pig ear, stored at –20°C and thawed within 6 months for the experiments. On the day of the experiment, punches were cut out and hairs cut with scissors, as reported previously by other authors (Gillet A *et al*., 2011). The outer skin surface (stratum corneum) and the inner region (dermis) were used for the experiments. After drying, the skin was cut into circles of 3 cm diameter. For permeation experiments, the skin was mounted in the Microglass Heim Franz diffusion cells. Briefly, the porcine skin was mounted on the receptor compartment of a Franz diffusion cell assembly with the stratum corneum side facing upwards into the donor compartment. Seven milliliters of 3:7 (v/v) CH_3CH_2OH:pH 7.4 PBS was used as the receptor medium. A measured amount of transfersomes or ethosomes containing VK1 were poured or nebulized into the donor compartment. The concentration of all samples was adjusted to achieve the same VK1 amount (1 mg) in the donor compartment. The available diffusion area between compartments was 0.6 cm^2. The Franz cells were mounted on a H+P Labortechnik Variomag Telesystem (Munchen, Germany) and placed in a thermostatic bath (Haake DC30; Thermo Fisher Scientific, Waltham, MA, USA). The experiments were carried out at a stirring rate of 600 rpm and temperature of 37°C. At predetermined time frames, 700 µL of the receptor phase were withdrawn and replaced with the same amount of fresh medium. The amount of VK1 in the withdrawn samples was determined by HPLC. At the end of the experiments, skin surfaces were thoroughly washed with distilled water to remove the excess of formulation. Epidermis and dermis were then separated by heating and then placed in separate eppendorfs. Finally, the VK1 accumulated in epidermis or dermis was extracted with 1 mL of CH_3CN by bath sonication (Branson 3510) five times for 30 minutes. The CH_3CN phase was filtered using 0.45 µm membranes, and the resulting filtrate was analyzed by HPLC to determine the VK1 content. The amount of VK1 accumulated in the different layers of the skin was calculated as the ratio of amount of VK1 (ng) to weight of epidermis or dermis (mg), (Da Silva A., 2012; Lopes L.B. *et al*., 2007).

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

At the end of the penetration experiments (see above), the pig skin was dismounted and frozen at -20°C. Before use, the sample was thawed at room temperature and the spectra were collected over the wavenumber 4000–450 cm^{-1} region by a Bruker Alpha-P spectrometer (128 scans; 4 cm^{-1} resolution), equipped with an attenuated total reflection diamond crystal accessory (Bruker Optics Inc., Germany). The raw data were elaborated by ATR correction (OPUS software, Bruker Optics Inc., Germany) and analysed by Origin Pro (OriginLab, US). The band maxima were assigned by second derivative. Fourier self-deconvolution (FSD) of the overlapping band components (hidden peaks) were resolved by the second-order derivative with respect to the wavelength after smoothing with a nine-point Savitsky−Golay function. Deconvolution was performed using Gaussian line shape. A nonlinear least-square method was used to take the reconstituted curve as close as possible to the original deconvoluted spectra. The fitting results were further evaluated by examining the residual from the differences between the fitted and the original curve and accepted when the regression coefficient was higher than 0.999.

Statistical analyses

All experiments were carried out at least in triplicate and the results are expressed as mean ± standard deviation. The comparison of the samples was performed by one-way ANOVA followed by Bonferroni-Holm post-analyses (Daniel's XL Toolbox 6.53 for Excel). The level of significance was taken at $p<0.05$.

Confocal laser scanning microscopy (CLSM) experiments

The lipophilic fluorescent probe PE-CF was added (2% w/w of the total lipids) in the organic phases during the preparation of the vesicles to prepare fluorescently labeled nanocarriers. Subsequently, fluorescent vesicles were applied onto excised porcine skin mounted on Franz Cells in the same conditions previously described. After

24 h of treatment the skin samples were washed and frozen at -80 °C. The cross-sectional tissue samples of skin treated with PE-CF-nanocarriers were prepared using a cryomicrotome (CM1850, Leica Microsystems GmbH, Wetzlar, Germany). Sections of skin of 30 μm thickness were cut with a cryostat microtome longitudinally to the skin strata to investigate the fluorescent probe distribution in the different skin strata. Analyses were carried out using confocal laser scanning microscopy (CLSM) (LSM 510 Zeiss confocal inverted microscope). A 5× objective lens (Zeiss) and an Argon laser were used (λ ex = 488 nm). Fluorescence images of skin samples were obtained after CLSM calibration and the fluorescent signal detected in skin cross-sections was converted to normalized concentration by verifying that the coincidence between fluorescence ratio and concentration ratio. The concentration was normalized with respect to the maximum concentration detected across stratum corneum axis. Likewise, the non-dimensional length was defined as $x = x/L$, where x is the stratum corneum abscissa and L is the depth of stratum corneum. The final profiles were averaged on at least 20 profiles.

Results

Characterization of the vesicles containing VK1

This study was focused on the development of formulations based on different types of nano-sized lipid vesicles, i.e. liposomes, transfersomes and ethosomes to investigate their effect on VK1 accumulation into the skin and on transdermal VK1 delivery. All the formulations were characterized in terms of size, ZP, soon after preparation and upon storage at 4 °C. The results are reported in Table 1. In the case of liposomes, the mean diameter was about 138.4 nm soon after preparation, without any significant change after 60 days; after 6 month of storage, a slightly increase of the liposome mean diameter was found, namely 146.7 nm. However, it is worthy of note that the SD after 60 days is quite large, about 20 nm, suggesting that the increase of the mean diameter after 6 months could also be considered not significant. The PI of these carriers was 0.2 after preparation and remained unaffected after 6 months of storage at 4 °C. Liposomes showed a negative ZP of about -13 mV without any significant differences during storage. Transfersomes showed a lower mean diameter, about 89.7 nm, without significant differences following storage at 4 °C; in fact after 60 days the diameter of the vesicles was 93.6 and remained very stable (95.3 nm) after 6 months. On the contrary, the Trans PI increased from 0.1 to 0.2 after 60 months as well as the ZP that was always negative but increased of about 10 mV after 6 months of storage. In the case of ethosomes the different methods of preparation significantly affected ethosomes size distribution. In fact, Etho2 had a low mean diameter of about 86.9 nm soon after preparation, while the same vesicles prepared without the extrusion step (Etho1) had a mean diameter of about 112.1 nm. For both the formulations but especially for Etho1, the mean diameter increased after 60 days of storage at 4 °C (97.1 and 143.1 nm for Etho1 and Etho2 respectively) but did not changed until 6 months. Furthermore, Etho2 and Etho1, showed a low PI of about 0.1, without any significant difference during storage. Finally, the ZP was always negative and comparable after 6months of storage at 4 °C.

Formulation	After preparation			After 60 days			After 6 mounth		
	Mean diameter (nm ± SD)	PI ± SD	PZ ± SD	Mean diameter (nm ± SD)	PI ± SD	PZ ± SD	Mean diameter (nm ± SD)	PI ± SD	PZ ± SD
Lipo	138.4 ± 0.77	0.230 ± 0.0	-13.5 ± 4.1	138.0 ± 19.9	0.240 ± 0.0	-11.4 ± 6.1	146.7 ± 15.5	0.256 ± 0.0	-13.0 ± 0.5
Trans	89.7 ± 6.0	0.149 ± 0.0	-12.3 ± 2.0	93.6 ± 3.8	0.203 ± 0.0	-16.5 ± 1.0	95.3 ± 4.4	0.210 ± 0.0	-22.6 ± 5.4
Etho1	112.1 ± 5.8	0.102 ± 0.0	-18.3 ± 1.6	143.1 ± 8.0	0.109 ± 0.1	-20.4 ± 3.7	143.9 ± 3.4	0.113 ± 0.0	-16.2 ± 1.6
Etho2	86.9 ± 2.4	0.113 ± 0.0	-12.5 ± 2.6	97.1 ± 5.5	0.118 ± 0.1	-18.3 ± 3.5	99.8 ± 7.4	0.118 ± 0.0	-16.3 ± 0.5

Table 1. Characteristics of the formulations.

To verify if the formulations were suitable to be administered on the skin in form of aerosol, the developed suspensions containing the different nanocarriers were nebulized through a portable device. The technological characteristics of the nanocarriers were checked before and after nebulization. An image of aerosol produced by nebulization of liposomes, but representative of all the formulations, is shown in Figure 1.

Figure 1. Nebulization of the formulation by a portable device.

The characteristics of liposomes, ethosomes and transfersomes before and after nebulization through the device are reported in Table 2. For all the considered

formulations, any significant alteration of samples size was observed following nebulization.

Formulation	Mean diameter (nm ± SD) BN	PI ± SD BN	Mean diameter (nm ± SD) AN	PI ± SD AN
Lipo	139.5 ± 3.5	0.2 ± 0.2	140.8 ± 4.8	0.2 ± 0.1
Trans	86.7 ± 5.4	0.1 ± 0.2	87.4 ± 6.8	0.3 ± 0.1
Etho1	113.85 ± 0.9	0.1 ± 0.00	117.55 ± 5.86	0.1 ± 0.00
Etho2	92.7 ± 1.80	0.1 ± 0.03	95.7 ± 3.14	0.1 ± 0.01

BN: before nebulization; AN: after nebulization.

Table 2. Mean diameter and polydispersity index before and after nebulization of the formulations.

In the following step of the work, the ability of the nanocarriers to pass through a polycarbonate membrane with a pore diameter of 50 nm, smaller than the vesicle size, was investigated. In this experiment, deformability of transfersomes and ethosomes was compared with that of liposomes, which were considered the control. This experiment should provide useful information on how the surfactant, i.e SC, and the ethanol used to prepare the vesicles, could affect the deformability of the vesicles, that could, in turn, facilitate the vesicle penetration into the skin. As shown in Table 3, liposomes presented a very low DI (about 29), with a strong reduction of the mean diameter of the vesicles if compared to the others formulations. In fact, in this case, the passage of the liposomes formulation trough the 50 nm membrane used for the test, led to a significate modification of the mean diameter of the vesicles that was reduced from about 148.6 nm to 99.3 nm. The deformability of transfersomes (about 290.3) was ten times higher if compared with that of liposomes. Compared with liposomes DI, also for Etho1 and Etho2 the DI was higher, namely about 79.6 and 171.4, respectively.

From the results summarized in Table 3, the deformability of these nanocarriers increased in the following order: Control < Etho1< Etho2< Trans.

Formulation	Mean diameter (nm ± SD) BE	Mean diameter (nm ± SD) AE	DI
Lipo	148.6 ± 3.5	99.3 ± 3.7	29.9 ± 2.5
Trans	85.3 ± 17.4	75.13 ± 9.8	290.3 ± 15.2
Etho1	117.3 ± 11.1	87.48 ± 5.5	79.6 ± 7.2
Etho2	92.73 ± 3.2	81.13 ± 2.1	171.4 ± 8.3

BE: before extrusion; AE: after extrusion.

Table 3. Determination of deformation index (DI) of vesicular suspensions

The amount of VK1 encapsulated into liposomes, transfersomes and ethosomes was determined by HPLC analysis and the results showed only one chromatographic peak, attributed to the VK1, suggesting that the phylloquinone was not altered during the preparation (data not shown). The VK1 actual loading of the different formulations is reported in Table 4. For each formulation, the actual VK1 encapsulation was higher than the theoretical one. In particular, in the case of Lipo, VK1 actual loading after preparation was about 214.6 µg/mg SPC. However, after 60 days of storage at 4°C a decrease of about 30 % was observed. Unexpectedly, a slight increase of the VK1/SPC ratio was observed after 6 months of storage (183.4 µg VK1/mg SPC). Concerning Trans, the VK1 actual loading was about 47.94 µg VK1/mg SPC and this value remained unaffected upon storage at 4°C. In the case of Etho2, only a slight and progressive decrease of the VK1 actual loading was found until 6 months of storage of the formulation at 4°C; in particular, the actual loading of VK1 was 211,7 µg VK1/mg SPC after preparation and 191 µg VK1/mg SPC after 6 months. Also in the case of Etho1, characterized by a VK1 actual loading lower than Etho2 after preparation (173.0 µg VK1/mg SPC), a slight and progressive decrease of the VK1 content into the vesicles during storage was observed.

		After preparation	After 60 days	After 6 months
Formulations	**Theoretical Loading (µgVK1/mg SPC)**	**Actual Loading (µgVK1/mg SPC ± S.D.)**	**Actual loading (µgVK1/mg SPC ± S.D.)**	**Actual Loading (µgVK1/mg SPC ± S.D.)**
Lipo	100	214.6 ± 0.1	152.0 ± 0.09	183.4 ± 0.02
Trans	20	47.94 ± 0.01	46.2 ± 0.1	45.7 ± 0.02
Etho-1	100	173.0 ± 0.01	165.7 ± 0.05	162.4 ± 0.03
Etho-2	100	211.7 ± 0.08	209.3 ± 0.07	191.0 ± 0.00

Table 4. VK1 encapsulation in the nanocarriers.

Stability studies

In the previous part of the study, VK1 was found to be altered following light exposure (see Chapter 1). Thus, the stability of the developed formulations during storage at different temperature (4 °C, 25°C and 40°C) was evaluated in absence of light. Visual inspections, organoleptic evaluation and characterization in terms of size and VK1 leakage during storage of the samples were carried out at different time frames. The results of the visual and organoleptic analysis are reported in Table 5. As shown from the results, for all the formulations stored at 25°C a marked odor was reported after the first 60 days, although not associated to the presence of aggregates. Moreover, all the formulations incubated at 40°C also developed a pronounced characteristic odor, associated to the presence of visible aggregates after 60 days. In addition, the color of the suspensions stored at temperatures higher than 4 °C changed from a milky to a more yellowish one.

Formulation	Storage condition	After preparation			After 60 days		
		Aspect	**Odor**	**Colour**	**Aspect**	**Odor**	**Colour**
Lipo	4°C	Uniform	Absent	Milky	Uniform	Absent	Milky
	25°C	Uniform	Absent	Milky	Uniform	Strong odor	Yellowish
	40°C	Uniform	Absent	Milky	Suspended material	Strong odor	Yellowish
Trans	4°C	Uniform	Absent	Milky	Uniform	Absent	Milky
	25°C	Uniform	Absent	Milky	Uniform	Strong odor	Yellowish
	40°C	Uniform	Absent	Milky	Suspended material	Strong odor	Yellowish
Etho-1	4°C	Uniform	Absent	Milky	Uniform	Absent	Milky
	25°C	Uniform	Absent	Milky	Uniform	Strong odor	Yellowish
	40°C	Uniform	Absent	Milky	Suspended material	Strong odor	Yellowish
Etho-2	4°C	Uniform	Absent	Milky	Uniform	Absent	Milky
	25°C	Uniform	Absent	Milky	Uniform	Strong odor	Yellowish
	40°C	Uniform	Absent	Milky	Suspended material	Strong odor	Yellowish

Table 5. Visual and organoleptic analysis of the formulations stored in different conditions.

Afterwards, Lipo, Trans, Etho1 and Etho2 were also characterized in terms of size and VK1 leakage during storage. As shown in Table 6, and in agreement with the visual and organoleptic analysis, liposomes stored at 40°C for 60 days, showed a marked increase of mean diameter (from about 136.8 nm to about 334.0 nm) and PI (from 0.2 to 0.5), confirming the presence of aggregates into the suspension. In addition, the VK1 actual loading strongly decreased of about 57% following alteration of the vesicles. In the case of Trans a marked increase of the mean diameter was not found (Table 7); however, an increase of the PI up to the value of 0.3, as well as a higher VK1 actual loading, were observed.

Storage	After preparation				After 60 days			
	Mean diameter (nm ± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)
4 °C					139.13 ± 12.5	0.2 ± 0.0	-11.4 ± 6.1	123.7 ± 4.9
25 °C	136.8 ± 1.7	0.2 ± 0.1	-13.5 ± 4.17	183.4 ± 0.1	175.8 ± 18	0.2 ± 0.1	-12.0 ± 2.4	132.7 ± 6.3
40 °C					334.0 ± 15.0	0.5 ± 0.2	-32.5 ± 3.6	78.8 ± 13

Table 6. Stability of Liposomes stored at different temperatures and in absence of light.

Storage	After preparation				After 60 days			
	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)
4 °C					85.4 ± 3.2	0.1 ± 0.0	-12.7 ± 10.4	25.4 ± 0.1
25 °C	84.0 ± 3.8	0.1 ± 0.03	-14.5 ± 1.6	47.31 ± 0.01	89.6 ± 7.9	0.1 ± 0.0	-4.1 ± 1.2	36.8 ± 0.0
40 °C					126.5 ± 15.2	0.3 ± 0.1	-11.5 ± 1.0	113.6 ± 0.2

Table 7. Stability of Transfersomes.

As shown in the Table 8 and 9, and in agreement with the visual and organoleptic analysis, ethosomes stored at 40°C for 60 days, showed a marked increase of mean diameter; in particular in the case of Etho1 the mean diameter increased from about 101 nm to about 277.1 nm and PI from 0.2 to 0.5 confirming the presence of aggregation of the suspension. In addition, the VK1 actual loading decreased of about 20%. In the case of the formulation Etho2, after storage at high temperature the mean diameter increased up to 284.8 nm and the actual loading was strongly reduced (about 60%). When Etho1 and Etho2 were stored at 25 °C, the vesicles size significantly increased; the alteration of Etho1 vesicles was also associated to a significant increase of VK1 actual loading, that is a clear sign of a lipid loss. On the contrary, for Etho2 this

phenomena was not observed. Therefore, all the formulation developed did not undergo further characterizations under these storage conditions, i.e. 40 and 25°C. It's worthy of note that, in all the samples stored at the temperature of 4 °C, any effect on the suspension stability was found.

Storage	After preparation				After 60 days			
	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)
4 °C					133.5 ± 2.8	0.1 ± 0.08	-15.0 ± 1.0	82.91 ± 0.7
25 °C	101 ± 1.9	0.1 ± 0.1	-12.3 ± 2.6	108.1 ± 0.1	126.4 ± 7.2	0.2 ± 0.07	-19.0 ± 2.2	358.9 ± 0.2
40 °C					277.1 ± 4.4	0.5 ± 0.06	-33.0 ± 3.1	90.3 ± 0.0

Table 8. Stability of Etho1 stored at different temperatures and in absence of light.

Storage	After preparation				After 60 days			
	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)	Mean diameter (nm± SD)	PI ± SD	PZ ± SD	Actual VK1 encapsulation (µg/mg SPC ± SD)
4 °C					91.8 ± 3.1	0.2 ± 0.1	-13.6 ± 0.4	108.6 ± 6.8
25 °C	84.4 ± 3.7	0.1 ± 0.0	-10.0 ± 3.9	125.5 ± 0.1	107.2 ± 5.1	0.1 ± 0.1	-19.0 ± 3.1	78.6 ± 17.2
40 °C					284.8 ± 12.5	0.2 ± 0.2	-24.3± 9.5	50.8 ± 35.2

Table 9. Stability of Etho2 stored at different temperatures and in absence of light.

Ex vivo experiments

The accumulation/permeation of VK1 in/through the porcine skin were investigated on Franz cells. In the receptor compartment, VK1-encapsulating nanocarriers were used as such or nebulized through the medical device Eauté. In Figure 2, the amount of VK1 found in the epidermis following 24 h of skin incubation with the different formulation is shown.

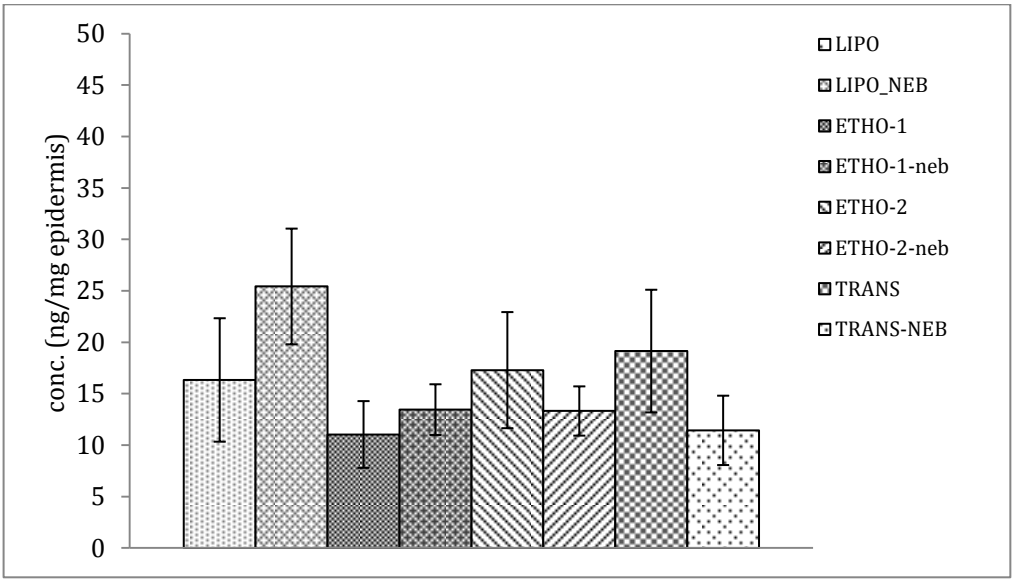

Figure 2. Accumulation of VK1 in epidermis. Legend: Lipo: liposomes containing VK1, administrated as such in the donor compartment. Lipo-NEB: liposomes containing VK1, administrated in form of aerosol in the donor compartment. Etho1: ethosomes containing VK1, administrated as such in the donor compartment. Etho1-NEB: ethosomes containing VK1, administrated in form of aerosol in the donor compartment. Etho-2: ethosomes containing VK1, administrated as such in the donor compartment. Etho-2-NEB: ethosomes containing VK1, administrated in form of aerosol in the donor compartment. Trans: transfersomes containing VK1, administrated as such in the donor compartment. Trans-NEB: transfersomes containing VK1, administrated in form of aerosol in the donor compartment.

The amount of VK1 accumulated into the epidermis was about 16, 11, 17 and 19 ng/mg of epidermis with Lipo, Etho1, Etho2 and Trans respectively. In the case of Lipo the nebulization of the formulation slightly increased the accumulation of VK1 (about 25 ng/mg of epidermis). On the contrary the use of the nebulized form of Etho1 did not

significantly affect the VK1 accumulation (13.4 ng/mg of epidermis) that remained very similar to that obtained in the skin treated with the same formulation used as such. On the contrary, in the case of Trans, and Etho2, the nebulization of suspension slightly reduced the VK1 accumulation in the epidermis, if compared with the formulations tested ad such (11.43 and 13.3 ng/mg of epidermis for Trans-NEB and Etho2-NEB, respectively).

The amount of VK1 found into the dermis is shown in Figure 3. The use of ethosomes or transfersomes led to an increase of the VK1 accumulated into the deeper layers of the skin, compared with Lipo. In particular, we found that Etho1 and Etho2 led to strong increase of VK1 accumulation compared to Lipo (51.3 and 40.8 vs 24.6 ng/mg of dermis, respectively). In the case of Trans administered as solution, a higher improvement of the VK1 accumulation into dermis was found (60.3 ng/mg of dermis). The use of the nanocarriers by nebulization on the skin significantly affected the VK1 accumulation in the dermis. In particular, the nebulization of Lipo led to a decrease of VK1 in the dermis of about 25%, compared with the same formulation administered as liquid; in all the other cases, the aerosol form improved the accumulation of VK1. In particular, Etho1-NEB and Trans-NEB led to an increase of amount of VK1 accumulated into the dermis that was higher than the 50% compared with Etho1 and Trans.

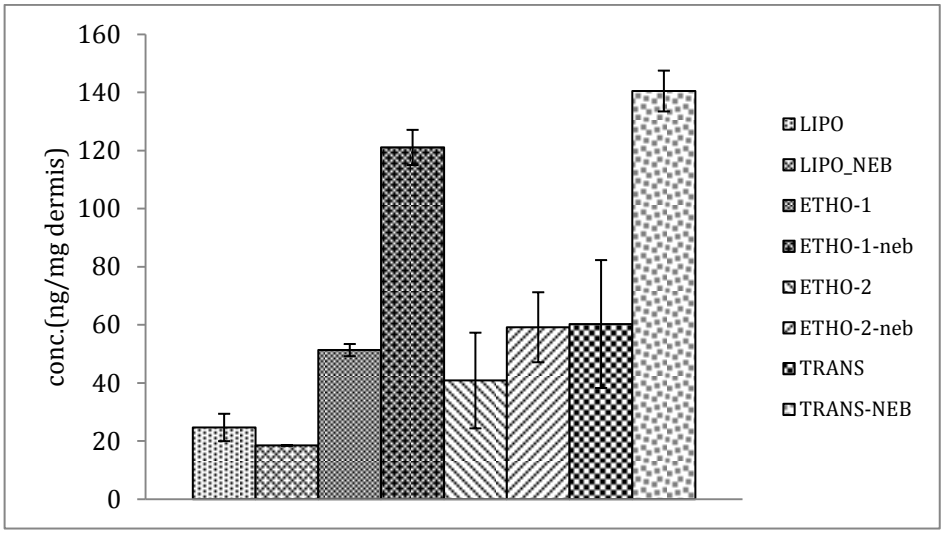

Figure 3. Accumulation of VK1 into the dermis. Legend: Lipo: liposomes containing VK1, administrated as such in the donor compartment. Lipo-NEB: liposomes containing VK1, administrated in form of aerosol in the donor compartment. Etho1: ethosomes containing VK1, administrated as such in the donor compartment. Etho1-NEB: ethosomes containing VK1, administrated in form of aerosol in the donor compartment. Etho2: ethosomes containing VK1, administrated as such in the donor compartment. Etho2-NEB: ethosomes containing VK1, administrated in form of aerosol in the donor compartment. Trans: transfersomes containing VK1, administrated as such in the donor compartment. Trans-NEB: transfersomes containing VK1, administrated in form of aerosol in the donor compartment.

No permeation rate was observed with conventional liposomes encapsulating VK1 during 24h of incubation (data not shown). On the contrary, in the case of transfersomes and ethosomes, an enhanced permeation rate was observed, dependently on the formulation and on the mode of administration (as such or in nebulized form). As reported in Figure 4, when using Trans, VK1 penetrated gradually and linearly with about 64% of the total VK1 administered permeated after 24h of incubation. On the other hand, in the case of Etho1, the penetration of VK1 increased with the time reaching the 100% in the receiving cell after 24 h. A lower permeation rate was found for Etho2; in particular, VK1 permeation was observed after just 30 minutes, but about 37% of the applied dosage penetrated in the receptor compartment after 24 h of incubation.

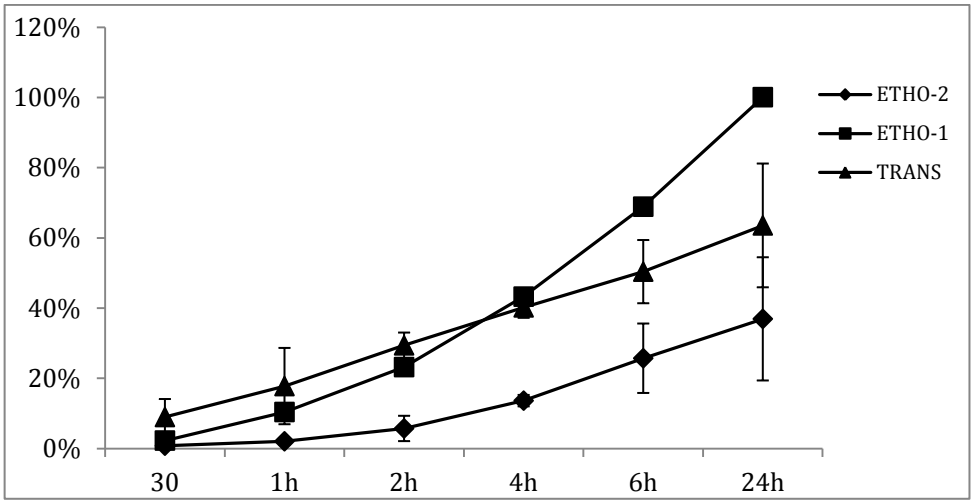

Figure 4. Permeation of Etho-1, Etho-2 and Trans .Legend: Etho1: ethosomes containing VK1 administrated as such in the donor compartment, Etho2: ethosomes containing VK1 administrated as such in the donor compartment, Trans: transfersomes containing VK1 administrated as such in the donor compartment.

Permeation profile of VK1 obtained with the formulations tested in the nebulized form is reported in Figure 5. When trans was applied in the aerosol form on the skin, the permeation profile of VK1 was completely different if compared to Trans; in particular, a low permeation profile was found, reaching only about 6% after 24 h. Also, in the case of Etho1-NEB the permeation profile was lower, if compared with Etho1; however, in this case, a relatively high VK1 permeation rate was found (about 67% after incubation for 24h). Finally, the use of Etho2-NEB brought to a VK1 permeation profile very similar to that obtained with Etho2 (33% and 36% of VK1 penetrated after 24h).

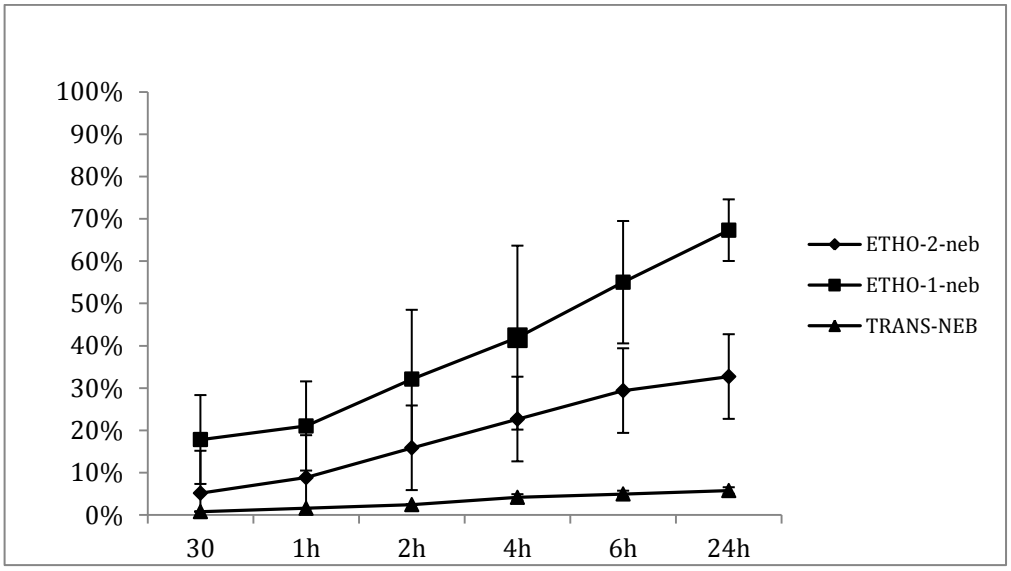

Figure 5. Permeation of Etho-1-neb, Etho-2-neb and Transf-neb. Legend: Etho1-NEB: ethosomes containing VK1 and administrated in form of aerosol in the donor compartment. Etho2-NEB: ethosomes containing VK1 and administrated in form of aerosol in the donor compartment. Trasf-NEB: transfersomes containing VK1 and administrated in form of aerosol in the donor compartment.

ATR FTIR spectroscopy

The possible effects of the treatment with the lipidic vesicles on the organization of the major constituents of the stratum corneum was assessed by ATR-FTIR spectroscopy, using the spectrum of untreated human epidermis mounted on Franz cell for 24 h. Possible alterations of spatial organization of lipidic lamellae were verified by analysing CH_x stretching region (2988-2828 cm^{-1}). As reported in Table 10, the second derivative of this region evidenced four different peaks of which the asymmetric ($\upsilon_{asym}CH_2$) and symmetric ($\upsilon_{sym}CH_2$) stretching bands centered at about 2920 and 2850 cm^{-1} are the most relevant. Generally speaking, the treatment with the different type of vesicles, namely liposomes, ethosomes and transferosomes, does not determined any significant shift of these two bands (ANOVA, $\upsilon_{sym}CH_2$: p=0.083; $\upsilon_{sym}CH_2$: p=0.051) suggesting that their penetration does not affect the conformation of the stratum corneum lipids(see table 10).

Sample	$\upsilon_{sym}CH_2$	$\upsilon_{asym}CH_2$	$H_{2920/2850}$	*FWHM*
Untreated	2851,91 ± 1,09	2924,61 ± 0,00	10,561 ± 0,45	11,37 ± 1,25
Lipo	2853,63 ± 0,14	2925,11 ± 0,71	4,885 ± 0,00	10,18 ± 0,00
Lipo-VK1	2853,73 ± 0,00	2924,61 ± 0,00	5,058 ± 0,78	11,21 ± 1,18
Etho	2853,73 ± 0,00	2924,61 ± 0,00	4,542 ± 0,60	11,56 ± 0,38
Etho-VK1	2853,73 ± 0,00	2925,32 ± 1,00	6,965 ± 1,21	11,33 ± 0,50
Trans	2853,73 ± 0,00	2925,32 ± 1,00	4,851 ± 0,31	11,18 ± 0,28
Trans-VK1	2853,99 ± 0,37	2926,65 ± 0,88	5,338 ± 0,47	10,69 ± 0,21

Table 10 - Assignments and peak position of diagnostic bands of lipidic components of stratum corneum.

The deconvolution of $\upsilon(CH_x)$ region (Figure 6a) permitted to calculate the ratio between the maximum intensity of $\upsilon_{asym}CH_2$ and $\upsilon_{sym}CH_2$ ($H_{2920/2850}$), which is considered a measure of lateral interactions between acyl chains in the stratum corneum, and the area of the peak assigned to $\upsilon_{sym}CH_2$(A_{2850}) (Karande *et al*., 2005). The treatment of pig skin with the tested formulations determined a decrease of $H_{2920/2850}$values (ANOVA, p= 0.023) concomitant to an increase of A_{2850} values and these variations resulted significant also when subjected to the post hoc analyses (see table 10).

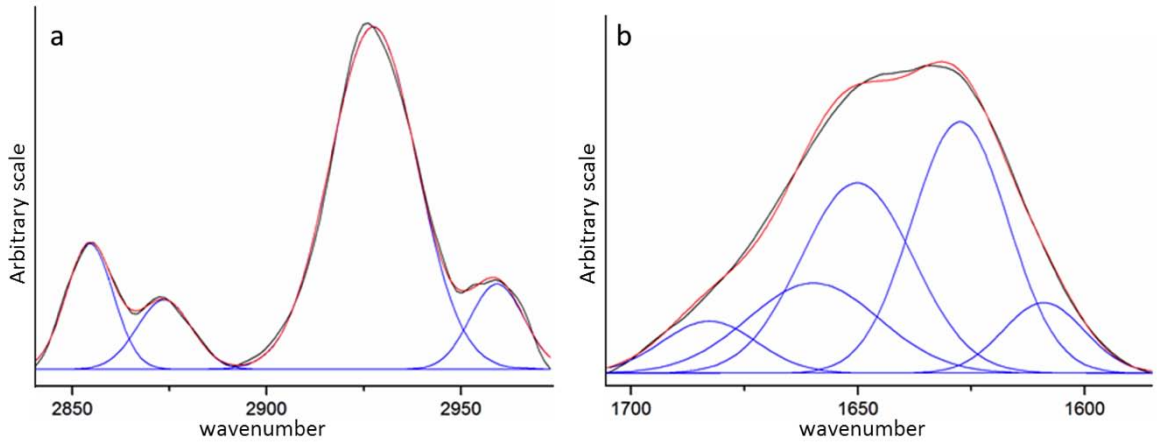

Figure 6. – Typical deconvolution bands of ATR-FTIR spectra in the (a) CHx stretching band and (b) Amide I regions. The original spectrum is depicted in black, the deconvoluted peaks are represented by the blue lines, red lines indicate the sum of deconvoluted spectrum.

The increase of the A_{2850} values can be justified considering that the penetration of the vesicles into the stratum corneum determined an enrichment of the lipidic components in the bilayer. The secondary effect of the vesicle partition within the stratum corneum is the formation of a more ordered structure measured by the significant decrease of the $H_{2920/2850}$ values with respect to the control. The lack of significant modifications of the lipidic lamellae structure of stratum corneum due to the treatment with the different types of vesicle is further confirmed by the lack of modifications of the methylene scissoring bandin the 1480-1430 cm^{-1} region. The lipids of stratum corneum can be packed in two different conformations hexagonal or orthorhombic and the CH_2 scissoring band width calculated from the second derivative of the spectrum (full width at 50% peak height, FWHM, see Table 10) is considered diagnostic of the extent of the two lipid phases in the stratum corneum (Damien *et al*., 2010). In all cases, FWHM values were close to 12.0 cm^{-1}indicating that lipids are organized in the most stable orthorhombic conformation (Damien *et al*., 2010). The other interesting band of the stratum corneum usually investigated for assessing the effect of permeant on the stratum corneum barrer properties is the peak assigned to the C=O stretching of the amide (Amide I) (Karande *et al*., 2005), which is the result of contributes of keratin conformations and presence of ceramides (*Cilurzo et al., 2014*). The deconvolution of this peak (Figure 6b) revealed the presence of five hidden peaks

assigned to the different conformation of keratins and the presence of ceramides (Table 11). Again, the treatment with the different formulations did not alter the ratios among the different hidden peaks expressed as percentages of the calculated areas. In conclusion, an enrichment of the lipidic component within the stratum corneum was noticed independently of the type of formulation/vesicle. The penetration vesicle does not affect the organization of the main components of the stratum corneum.

Wavenumber	Assignment	Area of the deconvoluted hidden peak (%)						
		Blank	**Lipo**	**Lipo-VK1**	**Etho**	**Etho-VK1**	**Trans**	**Trans-VK1**
1609	CER	8,36 ± 0,37	7,95 ± 0,03	7,68 ± 0,30	8,08 ± 0,46	7,76 ± 0,07	7,68 ± 0,19	7,88 ± 0,33
1627	β-sheet	35,96 ± 2,22	36,48 ± 0,02	36,95 ± 0,66	36,39 ± 1,10	37,17 ± 0,12	36,70 ± 1,47	36,76 ± 1,10
1650	random coil	31,08 ± 3,66	30,24 ± 0,04	30,19 ± 1,01	28,41 ± 1,40	29,40 ± 0,61	29,30 ± 1,13	31,40 ± 0,96
1659	α-elices	18,00 ± 1,77	18,60 ± 0,03	18,71 ± 0,08	20,20 ± 1,76	18,95 ± 0,51	19,56 ± 0,14	17,39 ± 1,32
1682	β-turn	6,60 ± 0,04	6,73 ± 0,06	6,97 ± 0,58	6,92 ± 0,27	6,73 ± 0,05	6,76 ± 0,28	6,57 ± 0,42

Table11 – Typical deconvolution bands of ATR-FTIR spectra in the (a) CHx stretching band and (b) Amide I regions. The original spectrum is depicted in black, the deconvoluted peaks are represented by the blue lines, red lines indicate the sum of deconvoluted spectrum.

CLSM experiments

Representative CLSM images of pig SC are shown in Figure 7.

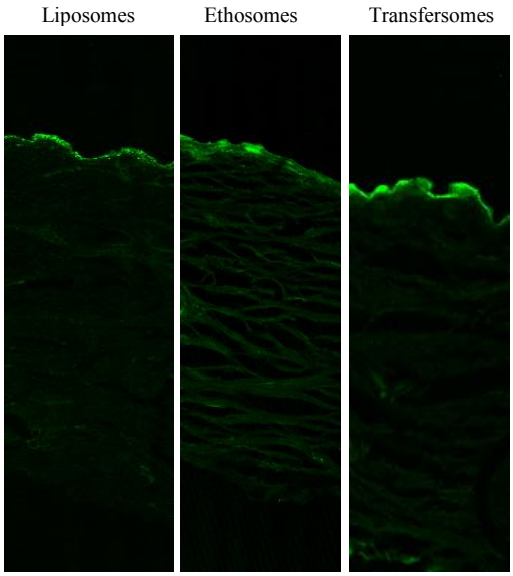

Figure 7: CLSM images of skin trated with liposomes, ethosomes and transfersomes.

In all cases, fluorescence acquisitions show that the fluorescence is prevalently localized in the external stratum corneum, while the signal rapidly decreases when going across the skin. From these images, a clear difference on fluorescence distribution between skin treated with liposomes and skin treated with the other two vesicle types can be observed. In particular, the case of transfersomes and ethosomes, although still limited to the outer layer of the stratum corneum, fluorescence appear distributed on a more extended surface. However, to quantify differences among fluorescence distribution into the skin section treated with the different fluorescently labeled nanocarriers, the analysis of fluorescence distribution profile was carried out. In figure 10, the fluorescence intensity versus the skin depth is reported. In the figure, we observe a fluorescence profile that decrease very rapidly for skin treated with all the formulations. However, a more rapid decrease of the fluorescence profiles is observed in the skin treated with liposomes, while the initial slope of the profiles is clearly lower

for ethosomes and transferosomes (Figures 8). From these data is not possible to observe a net difference between the fluorescence profile into the skin treated with ethosomes and transfersomes.

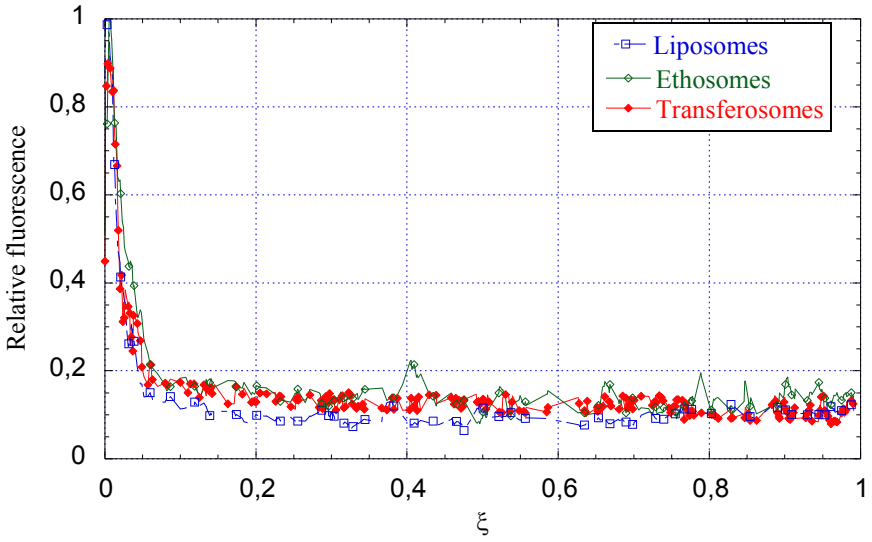

Figure 8: Concentration profile at 24 h of liposomes, ethosomes and transfersomes.

Discussion

The main goal of this part of the work was the development of aqueous formulations for topical administration of VK1 that could optimize the VK1 penetration into the skin. However, in line with the general scope of this experimental study, we developed aqueous formulations based on nanocarriers suitable to be administered on the skin by nebulization, in order to be proposed as an alternative to marketed formulations, e.g. Vigorskin®. For this reason, two types of vesicular systems, i.e. VK1-containing transfersomes and ethosomes were developed. Different studies demonstrated that transfersomes (Cevc, G., 1996) and ethosomes (Touitou E. *et al*., 2000) are able to improve the accumulation of actives into different layers of the skin and the penetration trough the skin. In the first part of the study, formulations based on transfersomes and ethosomes encapsulating VK1 were developed and characterized. For comparison purpose, a formulation based on liposomes encapsulating the VK1 was also prepared. It is worthy of note that, compared with the experimental procedures reported in the chapter 1, here liposomes were prepared by ethanol-injection method. A such method was chosen because, if compared to the "hydration of a lipid thin layer followed by extrusion" method previously used, is cheaper and certainly more suitable to undergo a scale-up process for future large scale production.

For all the nanocarriers, the technological characteristics of the vesicles before and after nebulization were investigated to verify the possibility to be administered in form of aerosol. Finally, the capability of these nano-sized lipid vesicles to enhance the accumulation or the permeation of VK1 into/trough the skin was evaluated. A first ethosomal formulation Etho1 has been prepared by the method typically used to prepare ethosomes and firstly described by Touitou (Touitou *et al*., 2000; Touitou E., US Patent N° 5,540,934, 1996; Dayan e Touitou, 2000); however, to investigate the effect of the size on the vesicle properties, ethosomes were also prepared by extrusion to obtain a smaller vesicle size. Finally, transfersomes were prepared as previously described (Scognamiglio *et al*., 2012). All the prepared formulations had a mean diameter < 150 nm, although the extrusion-sized ethosomes and the transfersomes were characterized by a mean size significantly lower compared to the other ethosomal formulation

(Etho1). All the prepared formulations presented a narrow size distribution as suggested by a polydisperisity index < 0.2. Moreover, all the vesicles were characterized by a high actual VK1 encapsulation, expressed as μg VK1/mg SPC, which was higher than the theoretical one; this suggested a significant lipid loss occurring during the vesicles preparation that was higher than the vitamin loss. This hypothesis can be supported by formation of vitamin-rich domains immiscible with the phospholipid bulk, as suggested in Chapter 1 and as previously hypothesized by our group and by other authors (Lopes LB, 2007; Campani *et al*., 2014). Moreover, in all the formulations, VK1 actual loading did not significantly change following storage at 4°C for 6 months. In order to propose these formulations for commercial development, the vesicle stability is certainly a crucial point to investigate. For all the formulations, mean diameter and size distribution did not changed following 6 months of storage at 4 °C. Moreover, no alteration of vesicles size, as well as of VK1 encapsulation, was observed following vesicles nebulization, suggesting the possibility to administer these formulations on the skin by nebulization, for example by the portable device Eauté. Previous studies demonstrated that the nebulization can alter ultraflexible vesicles, especially in the case of large mean diameters (Elhissi A.M.A. *et al*., 2012). However, in the case the reduced size of the developed nanovectors could justify their stability following the nebulization. Transfersomes and ethosomes have been proposed to deliver active molecules on the skin for their high deformability, compared to conventional liposomes (Cevc G. and Blume G., 2001; Touitou E., US Patent N° 5,540,934, 1996). The high deformability of these nanocarriers should make these vesicles the best candidate to delivery drugs also in the deepest layer of the skin (Cevc G., 1996; Godin B. *et al*., 2003). In our test on the vesicle deformability, conventional liposomes were considered the control. In this study, Trans showed the highest deformability, with a DI 10 times higher than that found in the case of liposomes. Also ethosomes DI was higher than liposomes DI, but with differences depending on the ethosome preparation method. In particular, Etho1 and Etho2 had a DI 2 and 5 times higher than liposome DI, respectively. These findings suggested the high deformability of transfersome-based formulation, reasonably due to the presence of an edge activator. In the case of ethosomes, DI value was lower for formulations characterized by a higher mean diameter. The deformability of ethosomes has been attributed to the presence of ethanol into the vesicles; thus, the sizing step used

in the case of Etho2 and carried out by extrusion could lead to a partial loss of ethanol with consequent lower vesicle deformability.

In a second part of the study described in this chapter, the stability of the developed formulation was investigated in different conditions of storage according to ICH guidelines (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use). We found that all the formulations were stable (no organoleptic, visible alteration or particle aggregation) up to 6 months of storage at the temperature of 4 °C. The same formulations showed visible aggregation after 60 days of storage at 40°C, but not at 25°C. On the other hand, a pronounced characteristic and marked odor was reported for samples stored at both 25°C and 40°C. In agreement with the visual and organoleptic analysis, the samples stored at 40°C showed a marked increase of mean diameter and PI, suggesting the presence of aggregates into the suspension; in the same conditions a reduction of VK1 actual loading was found, especially at the highest storage temperature. Vesicle instability could be ascribed to the lipid degradation, which can be enhanced when increasing the storage temperature (Pietzyk B *et al*., 2000, Grit M. *et al*., 1993). VK1 analysis by HPLC did not show the appearance of further chromatographic peaks, suggesting that the reduced VK1 actual loading should be due to the release of the encapsulated vitamin, and not to its degradation at 40 or 25°C. Taken together, these results suggest that these formulations could be proposed for further studies but considering a storage at 4°C of the final product.

Transfersomes and ethosomes have been largely proposed to enhance the accumulation in to thee skin and the transdermal delivery of drugs and active molecules. In fact, the presence of an “edge activator” or use of ethanol may enhance the deformability of the lipid bilayer (Cevc G., 1996; Godin B. *et al*., 2003). Moreover, in the case of ethosomes, the presence of ethanol also could act as penetration enhancer (Rao G.C., *et al*., 2008. Fang Y.P. *et al.*, 2008). Thus, the accumulation/permeation of VK1 into/through the skin when delivered by transfersomes or ethosomes was investigated and compared with that obtained with conventional liposomes. In the *ex-vivo* experiments, both the ethosomes and transfersomes changed the VK1 accumulation in the skin, which, however, was different in the epidermis and dermis. The ATR FTIR

spectroscopy analysis evidenced an enrichment of the lipidic component within the stratum corneum independently of the type of formulation/vesicle used to treat the skin. Moreover, organization of the main components of the stratum corneum did not significantly changed following skin treatment with the different vesicles. Moreover, in these experimental conditions, any significant difference was found between skin samples treated with the different vesicles. These data suggest that vesicles do not change the lipid organization of the skin, while they can enrich the lipid composition of the outmost layer of the skin. Thus, it is possible hypothesize that the vesicles used in this study fuse with the stratum corneum where the lipids remain confined, while the VK1 is free to diffuse toward the deeper layer of the skin. CLSM studies, carried out by using fluorescently labelled lipids, confirmed that lipid components of the vesicles remain mainly located in the outer layers of the skin, although, in the case of ethosomes and transfersomes a more deep penetration was observed. Thus it is possible hypothesise that while liposomes only fuse with the stratum corneum, ethosomes and transfersomes, due to the ethanol and to the sodium colate, are able to penetrate more deeply into the epidermis where they disassemble leaving the VK1 the possibility to diffuse into the skin. In the case of ethosomes, if compared with liposomes, VK1 accumulation was increased into the dermis, with VK1 permeation into the acceptor compartment. The highest VK1 accumulation into the dermis was found with the formulation Etho1, suggesting that the highest deformability as well as a highest ethanol content (due to the absence of the extrusion step) could have a crucial role. An enhancement of VK1 accumulation/permeation into/through the skin was also observed with transfersomes. In these vesicles, vesicle permeation through the skin has been reported (Cevc *et al*., 1998).

Surprisingly, in the case of the formulation Etho1 and Trans, nebulization on the skin resulted in a dramatic enhancement of the VK1 accumulation into the dermis. However, in this study, nebulization did not show to change the technological characteristics of vesicles. It has been reported that ultraflexible vesicles are suitable to the nebulization, but also more susceptible to the alteration, and this effect has been attributed to the presence of the surfactant or ethanol (Elhissi A.M.A. et al., 2012). In this case, due to the very low diameter of the vesicles, the aerosolization does not destroy the carrier but could certainly “force” the deformation of the vesicles, that

could, in turn, favour the penetration into the outer layer of the skin. This hypothesis is supported by our findings in which we found that only in the case of the formulations Trans and Etho1, characterized by the highest deformability index, the nebulization led to a dramatic increase of VK1 accumulation into the skin. Further studies are necessary to support these hypothesis and to investigate the relation between the nebulization and enhanced performace of the developed formulation.

Conclusions

In this chapter, we investigated the possibility to prepare “elastic” vesicles (transfersomes) and ethanol-containing vesicles to increase VK1 accumulation into the skin. Different formulations of liposomes, transfersomes and ethosomes encapsulating VK1 for a topical administration by a nebulizer were designed and characterized. Firstly, we demonstrated that all these carriers are very stable at the temperature of 4°C for at least 6 months. Moreover, from the ex-vivo experiments the use of transfersomes and ethosomes, especially in the nebulized form, enhanced the accumulation of VK1 into the skin. Moreover, especially in the case of transfersomes, VK1 permeation through the skin was also observed. In conclusion, since the developed formulations were designed to be nebulized and to deliver an active compound on the skin, for a local effect in the deepest skin layers, transfersomes probably represented the formulation that, if administered in form of aerosol, could offer a good compromise between a high penetration into the skin and a limited permeation through it.

Chapter 3

Development of nanoemulsions for topical delivery of vitamin K1.

Abstract

In this third chapter, a new formulation to administer VK1 on the skin by nebulization is described. In particular, the work here described was focused on the development of a formulation with lower production cost, compared to the systems described in the previous chapters. Thus, a lipid-free aqueous formulation was designed. In particular, we developed a protocol by which nanoemulsions spontaneously form with a low-energy method. The developed nanoemulsions were stable during long time frames and can be nebulized. Finally, the developed VK1-containing nanoemulsions were tested on Franz Cells, showing an increased VK1 accumulation into the skin.

Keywords: Vitamin K1, nanoemulsions, Franz cell, low-energy methods.

Introduction

Emulsion-based delivery systems and in the particular nanoemulsions, represent a promising tool for the topical and transdermal administrations of drugs. nanoemulsions proposed for drug delivery were constituted by a fine oil/water dispersion with droplets having a size ranging from 100-600 nm (Nakajima *et al*., 1993; Nakajima, 1997). From a general point of view, oil-in-water nanoemulsions consist of two phases: an organic phase composed of an homogeneous oily solution and an aqueous phase. These systems are also often characterized by the presence of a hydrophilic surfactant and water–miscible solvent. These o/w nanoemulsions have found increasing use as delivery systems to encapsulate lipophilic bioactive components in cosmetic, personal care, functional food and pharmaceutical products (Saberi *et al*., 2012). Indeed, these formulations offer many advantages for the delivery of active compound into the skin (Sonneville-Aubrun *et al*., 2004); in fact, the presence of a surfactant (generally at a concentration in the range 3–10%), the large surface area of the internal oily phase, the low surface tension of the whole system as well as of the O/W interface, allow to enhance penetration of actives agents. Moreover, the small size of the droplets allows them to deposit uniformly on skin and facilitate the penetration of actives trough the skin layers; for all these reasons, nanoemulsions could be proposed to deliver active molecules onto the skin, as alternative to lipid-delivery systems such as liposomes and other vesicle-based systems (Bouchemal *et al*., 2004).

Nanoemulsions can be produced using different techniques usually classified as high-energy methods and low-energy methods. High-energy methods employ expensive equipments, such as high pressure valve homogenizers, microfluidizers, and sonicators (Mc Clements D.J. *et al*., 2011; Tadros *et al*., 2004). These mechanical devices are capable to disperse the oil into the aqueous phase in form of nano-droplets. On the other hand, the so-called “low-energy” methods included several approaches such as spontaneous emulsification, phase inversion temperature (PIT), and phase inversion composition (PIC) methods (Anton N. *et al*., 2008). Compared to the high-energy methods, the use of low-energy methods had some advantages in terms of scale-up and industrial transfer, since no expensive equipments are require. In addition, the

possibility to used non-aggressive features, can make these systems suitable to the delivery of labile molecule (Anton N. *et al*., 2009).

Aim of the work

In this study, we investigated the possibility to develop nanoemulsions containing VK1 using the low-energy method. The aim of this work was to produce a formulation for the administration of VK1 in the aerosol form using a very simply method that could represent a valid alternative in terms of cost of production compared to lipid-vesicles described in previous chapters. So, we prepared nanoemulsions by a spontaneous emulsification approach at room temperature using simple stirring and avoiding the use of expensive homogenization equipment. The influence of the physical properties of the oily phase, the surfactant nature and the effect of the oily phase/water phase ratio on the characteristics of the emulsion were studied in the first part of the work. Then, stability studies were carried out to establish the optimal storage conditions for the developed system. Finally, the possibility to administer VK1-containing nanoemulsions by nebulization and the capability of these formulations to facilitate VK1 accumulation into the skin layers were evaluated.

Materials and methods

Materials

Vitamin K1 (VK1) and α-tocopherol (αTOC) and were purchased from Sigma-Aldrich (St Louis, MO, USA). Analytical grade chloroform ($CHCl_3$) and ethanol (CH_3CH_2OH or EtOH) were obtained from Carlo Erba Reagents (Cornaredo, Italy). Polyoxyethilen-20-sorbitan-monooleate (tween 80) was provided by Farmalabor (Italy) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein-ammonium salt) (PE CF) was kindly gifted by Lipoid GmbH (Steinhausen, Switzerland).

Preparation of nanoemulsions

The preparation of nanoemulsions (NEs) was performed using the procedure based on spontaneous emulsification, previously described by Anton et al. (Anton *et al*., 2009), with some modifications. Briefly, the oil phase composed of αTOC and VK1 was mixed with the surfactant (tween 80) and the organic solvent (EtOH) with a vortex. Subsequently, the organic phase was added slowly into an aqueous phase placed under magnetic stirring at 700 rpm. The addition of the organic phase was performed using a syringe pump at the speed of 50µl/min (NE-300 "just infusion"™, new era pump system Inc., NY USA). Each sample was further stirred for 5 min at 1400 rpm. NEs were rapidly formed. Unless otherwise stated, the experiments were carried out using standardized conditions, namely 10 % (w/w) oily phase, 64 % (w/w) aqueous phase, surfactant 10 % (w/w).

Nanoemulsions size and zeta potential

Dimensional analysis was performed by photon correlation spectroscopy (PCS). For each sample, an aliquot of about 20µl was diluted in filtered water and analyzed by N5 (Beckman Coulter, USA). The average diameter and the size distribution of each

formulation were determined. The results were expressed as NEs mean diameter (nm) and polydispersity index (PI).

The zeta potential of NEs was performed by the Zetasizer Nano Z (Malvern, UK). Briefly, an aliquot of each sample (20µl) was diluted in filtered water and analyzed. The results were calculated by the average of the measurements obtained from three batches of the same formulation.

Stability studies

Physical stability of NEs was evaluated after different time frames on the formulations prepared at growing VK1 concentrations. Briefly, after preparation, each batch was stored at 4°C and at predetermined intervals, about 10 µl of the suspension was diluted in filtered distilled water and analysed by PCS, as reported above.

The formulations were tested in different storage conditions. In particular, NEs were incubated at different temperatures, namely 4, 25 and 40°C, in refrigerant (Hotpoint, Ariston, Italy) or in a laboratory oven (STF-F52Lt, Falc Instrument, Italy). All samples were held under nitrogen atmosphere and protecting them from the light. At predetermined intervals, the samples were analyzed in terms of physical appearance, odor, NEs size. NEs size was determined by PCS, as described above. For each formulation, the results were the mean of three different batches (n = 3).

NEs nebulization

NEs were also characterized before and after nebulization. To nebulize the formulations, a portable nebulizer (Eauté) kindly provided by Xenus, was used. Briefly, about 1 ml of the the suspension, previously characterized for size, was loaded into the device and nebulized by collecting the aerosol in a 20 ml glass vial. The collected suspension was then analyzed in terms of size.

Skin penetration experiments

The penetration of VK1 in the skin and its transdermal delivery were assessed with porcine ear skin. The porcine ears were kindly provided by a local slaughterhouse (Vendor Carni, Italy). All the experiments were performed on frozen-thawed skin used within 6 months. Full-thickness skin was removed from the dorsal side of the freshly excised pig ear, stored at 20 °C and used within 6 months. On the day of the experiment, punches were cut out and hairs cut with scissors, as already reported by other authors (Gillet A *et al*., 2011). The outer skin surface (stratum corneum) and the inner region (dermis) were used for the experiments. After drying, the skin was cut into circles of 3 cm of diameter. For permeation experiments the skin was mounted in a Franz diffusion cells (Microglass Heim, Italy). Briefly, the porcine skin was mounted on the receptor compartment of a Franz diffusion cell assembly with the stratum corneum (SC) side facing upwards into the donor compartment. Seven milliliters of 3:7 (v/v) ethanol-pH 7.4 PBS was used as the receptor medium. A measured amount of nanoemulsion containing VK1 was poured or nebulized into the donor compartment. Alternatively, a weighted amount of VigorSkinK1® cream was added into the donor compartment in contact with the excised skin. The concentration of all samples was adjusted to achieve same VK1 amount (1mg) in the donor compartment. The available diffusion area between compartments was 0.6 cm^2. The Franz cells were mounted on a H+P Variomag Labortechnik Telesystem (Germany) placed in a thermostatic bath Haake DC30 (Thermo Electron Corporation, Germany). The experiments were carried out at a stirring rate of 600 rpm and temperature of 37 °C. At predetermined time frames, 700 µl of the receptor phase were withdrawn and replaced with the same amount of fresh medium. The amount of VK1 in the withdrawn samples was determined by HPLC. At the end of the experiments skin surface were thoroughly washed with distilled water to remove the excess formulation. Epidermis and dermis were then separated by heating and then placed in separate eppendorfs. Finally, the VK1 accumulated in epidermis or dermis was extracted with 1 ml of CH_3CN for 5 times by bath sonicator (Branson 3510) for 30 minutes. The CH_3CN phase was filtered using 0.45 µm membranes and the resulting filtrate analyzed by HPLC to determine the VK1 content. The amount of VK1 accumulated in the different layers of the skin was calculated as ratio between amount (ng) of VK1/ weight (mg) of epidermis or dermis,

Da Silva A 2012; Lopes LB *et al*., 2007).

Confocal laser scanning microscopy (CLSM) experiments

The lipophilic fluorescent probe PE-CF was added (2% w/w of the total NEs) in the organic phases to prepare fluorescently labeled NEs. Subsequently, fluorescent NEs were applied onto excised porcine skin mounted on Franz Cells in the same conditions previously described. After 24 h of treatment the skin samples were washed and frozen at -80 °C. The cross-sectional tissue samples of skin treated with PE-CF-NEs were prepared using a cryomicrotome (CM1850, Leica Microsystems GmbH, Wetzlar, Germany). Sections of skin of 30 μm thickness were cut with a cryostat microtome longitudinally to the skin strata to investigate the fluorescent probe distribution in the different skin strata. Analyses were carried out using confocal laser scanning microscopy (CLSM) (LSM 510 Zeiss confocal inverted microscope). A 5× objective lens (Zeiss) and an Argon laser were used (λ ex = 488 nm). Fluorescence images of skin samples were obtained after CLSM calibration and the fluorescent signal detected in skin cross-sections was converted to normalized concentration by verifying that the coincidence between fluorescence ratio and concentration ratio. The concentration was normalized with respect to the maximum concentration detected across stratum corneum axis. Likewise, the non-dimensional length was defined as $x = x/L$, where x is the stratum corneum abscissa and L is the depth of stratum corneum. The final profiles were averaged on at least 20 profiles.

Results

Effect of nanoemulsions composition on particle size

The first phase of the work was aimed to investigate the influence of the NEs composition on the size of droplets obtained by spontaneous emulsification. In particular, different surfactant-emulsion ratios (SERs) were tested, maintaining the oily phase/aqueous phase (αTOC) ratio at 10% wt. The results of this first part of the formulative work are summarized in Table 1.

Formulation	% SER	O (%)	S (%)	W (%)	EtOH (%)	Diameter (nm)	IP	ZP (mV)
NE1	1	10	1	73	16	UD	UD	-34,906
NE2	5	10	5	69	16	250,4	0,973	-25,11
NE3	**10**	**10**	**10**	**64**	**16**	**209**	**0,107**	**-18,598**
NE4	12	10	12	62	16	263	0,239	-17,984
NE5	20	10	20	54	16	100,57	0,538	-15,082

Table 1: Different types of nanoemulsions designed. Legend: SER: surfactant/emulsion ratio; O: oily phase; S: surfactant; W:water, UD: undetectable**.**

NEs were obtained for all the surfactant/emulsion ratio, with the exception of the formulation NE1. In fact, using a low concentration of surfactant (1%), the mean diameter of the droplets was very high as well as the value of the PI was highest than 1. Thus, when the percentage of the surfactant was increased, the mean diameter was reduced. In particular a decrease from 250.4 nm in the case of the NE2 until to 100.57 nm for the formulation NE5 was observed. Moreover, only in the case of the formulation NE3 and NE4, a low PI (0.107 and 0.239, respectively) were obtained. In fact, for the formulation NE2 and NE5, the distribution of the droplets remained higher (0.973 and 0.538, respectively). The ZP of the NEs was always negative, but it decrease at increasing surfactant concentrations. In particular, for the formulations from NE1 to NE5, a ZP of -34.906, -25.11, -18.59, -17.984, and -15.082 mV were observed,

respectively. From the obtained results, the formulation NE3 was the NE with a good compromise between a small mean diameter and a narrow size distribution of the droplets; for this reason, NE3 was selected for the next steps of the study. Due to its lipophilicity, VK1 was added in the oil phase of the NE. In particular, in order to maintain the total oil phase at 10 % w/w, the amount of α-TOC was reduced and replaced with an equal amount of VK1.

In the second part of the study, different NEs with increasing concentration of the active compound were developed. The different VK1-containing nanoemulsions (NEs-VK1) developed are shown in table 2. All the NEs developed had a mean diameter, after preparation, ranging from about 200 to 250 nm; in particular, in the case of NEs prepared without the inclusion of the active compound, the diameter of the droplets was lower (about 201.2 nm) compared with NEs-VK1. It is worthy of note that, although the presence of VK1 in the formulations led to an increase of the droplets size, a direct correlation between the amount of VK1 used and the effect on the droplet mean diameter was not found (see table 2). The addition of VK1 in the formulations also affected the PI, with an increase from 0.14 to 0.23-0.25, which was independent on the amount of VK1 used. Finally, the ZP was negative and very similar for all the formulations (-14.6, -15.6, -13.7, and -13.4 for NEs, NEs-VK1 5%, NEs-VK1 3%, and NEs-VK1 1%, respectively). After 30 days of storage at 4 ºC, all the VK1-containing formulations did not show any significant differences in terms of mean diameter of the droplets, PI and ZP; these characteristics of the nanoemulsions did not significantly changed also after 3 months of storage at 4°C.

		After preparation			After 30 days			After 3 months		
Formulation	**VK1 %**	**Mean diameter (nm± SD)**	**PI ± SD**	**ZP ± SD (mV)**	**Mean diameter (nm± SD)**	**PI ± SD**	**ZP ± SD (mV)**	**Mean diameter (nm± SD)**	**PI ± SD**	**ZP ± SD (mV)**
NEs	-	201.2 ± 5.5	0.14 ± 0.03	-14.6 ± 2.4	200 ± 8.9	0.22 ± 0.13	-11.9 ± 2.26	211 ± 3.35	0.17 ± 0.21	-11.0 ± 3.57
NEs-VK1 5%	5	222 ± 13.5	0.24 ± 0.16	-15.6 ± 1.4	217.3 ± 8.2	0.30 ± 0.02	-14.8 ± 3.84	219.5 ± 4.8	0.32 ± 0.01	-15.36 ± 1.95
NEs-VK1 3%	3	265.4 ± 4.2	0.23 ± 0.01	-13.7 ± 2.7	260.3 ± 3.0	0.25 ± 0.01	-15.2 ± 0.01	260.2 ± 4.52	0.25 ± 0.02	-14.4 ± 2.08
NEs -VK1 1%	1	246.9 ± 1.9	0.25 ± 0.04	-13.4 ± 0.2	258.9 ± 6.3	0.25 ± 0.01	-14.6 ± 0.83	258.0 ± 9.5	0.25 ± 0.05	-14.0 ± 1.79

Table 2. Characteristics of the VK1-containing nanoemulsions.

Taking into account that the NEs-VK1 was designed to be administrated as aerosol, using a portable device, the possibility to nebulize these nanoemulsions were checked. In particular, the diameter of the droplets before and after the passage through the device was studied. The results evidenced that all the formulations could be administrated in the nebulized form since the technological characteristics of all NEs did not significantly change following aerosolization (Table 3).

Formulation	**Mean diameter (nm ± SD) BN**	**PI** ± SD **BN**	**ZP** ± SD **(mV) BN**	**Mean diameter (nm ± SD) AN**	**PI** ± SD **AN**	**ZP** ± SD **(mV) BN**
NEsVK1 5%	226.0 ± 5.4	0.23 ± 0.02	-13.9 ± 0.07	233.2 ± 0.2	0.26 ± 0.02	-15.4 ± 0.1
NEs-VK1 3%	257.5 ± 0.3	0.22 ± 0.05	-13.1 ±0.10	259.4 ± 4.1	0.19 ± 0.14	-16.6 ± 1.7
NEs -VK1 1%	239.0 ±6.5	0.25 ±0.00	-12.8 ± 0.4	241.0 ± 9.4	0.22 ± 0.03	-18.6 ± 1.7

BN: before nebulization; AN: after nebulization.

Table 3. Mean diameter and polidispersity index before and after nebulization of the NEs.

Stability studies

In our previous formulative studies, the stability of the developed formulations following storage of the samples in different conditions in the absence of light was evaluated (see Chapter 1 and 2). In this part of the study, the stability of the NEs-VK1 was evaluated in the same conditions previously used, namely at the temperatures of 4 °C, 25°C and 40°C in absence of light. Visual inspections, organoleptic evaluation and characterization in terms of mean diameter, PI and ZP during storage of the samples were carried out at different time frames. The results of the organoleptic analyses are reported in Table 4.

Formulation	Storage condition	After preparation			After 30 days			After 3 months		
		Aspect	Odor	Colour	Aspect	Odor	Colour	Aspect	Odor	Colour
NEs-VK1 5%	4 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
	25 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
	40 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
NEs - VK1 3%	4 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
	25 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
	40 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
NEs - VK1 1%	4 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
	25 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish
	40 °C	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish	Uniform	Absent	Yellowish

Table 4. Organoleptic analyses of NEs-VK1.

The results carried out from the visual inspection and the organoleptic evaluations demonstrated that all the nanoemulsions containing VK1 were stable at all the storage conditions (Table 4). In particular, any alteration in terms of colour, odor and presence of aggregates were found after 3 months of storage of the samples at the temperature of 4 °C but also at higher temperatures (storage at 25 and 40 °C). Furthermore, in Table 5, the characterizations of these NEs-VK1 are reported.

In the case of storage of NEs-VK1 5% at the temperature of 4 °C, the size of the droplets and the values of PI and ZP did not shown any variation; in addition, after 3 months of storage of the samples at 25 °C the mean diameter and the PI increased of about 10% and 30%, respectively. On the contrary, after 3 months of storage of NEs-VK1 5% at 40 °C, only an increase of about 26% of the mean diameter of the droplets after 30 days of storage was observed; this value of mean diameter remained stable after 3 months of storage. The PI and ZP remained unchanged during storage. Concerning NEs-VK1 3%, the storage of 4 and 25°C did not lead any significant change of the formulation characteristics. On the contrary, after 3 months of storage of NEs-VK1 3% at 40 °C, an increase of about 20% of the diameter of the droplets was observed but not

correlated with an enhancement of the PI. Finally, in the case of NEs-VK1 1% after 30 days of storage of the formulation at 4°C a slight increased of the mean diameter, from 232.2 to 253.9 nm, was observed; after that any further increase was found in the formulation until 3 months of storage. Instead, after storage of the formulation at higher temperature, the increase of the mean diameter of NEs was of about 11% and 24% respectively in samples stored at 25 and 40 °C, which remained then stable for further 2 months of storage. Moreover, any variation in PI and ZP was not observed.

Formulation	Storage	After preparation			After 30 days			After 3 months		
		Mean diameter (nm±SD)	PI ± SD	PZ± SD	Mean diameter (nm±SD)	PI ± SD	PZ ± SD	Mean diameter (nm±SD)	PI ± SD	PZ ± SD
NEs-VK1 5%	**4 C°**	215.7±2.33	0.23±0.02	-14.14±0.29	214.9 ± 7.63	0.26 ± 0.04	-17.44±0.12	219± 4.80	0.32 ± 0.0	-14.8 ± 2.6
	25 C°				227.3 ± 3.74	0.31 ± 0.03	-16.87±0.15	238.3±4.10	0.32 ± 0.01	-14.8± 2.91
	40 C°				288 ± 8.48	0.20 ± 0.11	-13.79±0.11	283± 21.2	0.39 ± 0.05	-14.44±0.60
NEs -VK1 3%	**4 C°**	254.8±10.74	0.28±0.09	-14.89±2.68	255 ± 3.46	0.302 ± 0.07	-14.73±0.68	260.3±4.69	0.30 ± 0.10	-14.79±0.75
	25 C°				260.35±5.72	0.255 ± 0.09	-14.43±0.176	262.15±5.72	0.25 ± 0.1	-14.43±0.17
	40 C°				318.2 ± 2.47	0.296±0.097	-13.43±0.25	358± 14.1	0.29 ± 0.00	-11.5±1.42
NEs- VK1 1%	**4 C°**	232.2±18.7	0.254±0.07	-14.00±1.01	253.9±6.15	0.241±0.05	-14.37±0.44	254.1 ± 3.95	0.24±0.00	-13.9±1.51
	25 C°				260.6±6.15	0.23±0.165	-14.74±0.20	260.6 ± 6.15	0.23±0.16	-14.7±0.20
	40 C°				304.2±9.04	0.238±0.166	-13.65±0.84	342.3 ± 11.8	0.25±0.14	-12.77±1.11

Table 5. Stability studies of NEs-VK1.

Ex vivo experiments

The VK1 accumulation/permeation into/through the skin treated with the NEs-VK1 was investigated in *ex-vivo* experiments using Franz cells. These experiments demonstrated that the use of NEs can be successfully used as alternative to the vesicle-based systems described in the previous chapters. Further information on this part of the

experimental work have been omitted. Confidential treatment has been requested with respect to the omitted portions.

CLSM experiments

Representative CLSM images of pig stratum corneum and dermis are shown in Figure. 1.

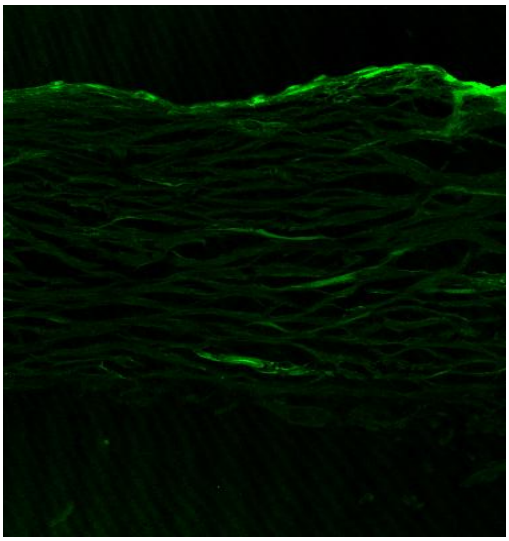

Figure 1: CLMS image of skin treated with nanoemulsions.

Fluorescence acquisitions show that the fluorescence is prevalently localized in the external stratum corneum region, while the signal rapidly decreases when going across stratum corneum sections. However, to underline the fluorescence distribution into the skin section treated with NEs, the analysis of fluorescence distribution profile was carried out. In figure 2, the fluorescence intensity versus the skin depth is reported.

In the figure, we observed a fluorescence profile that decrease very rapidly for skin treated with NE formulation.

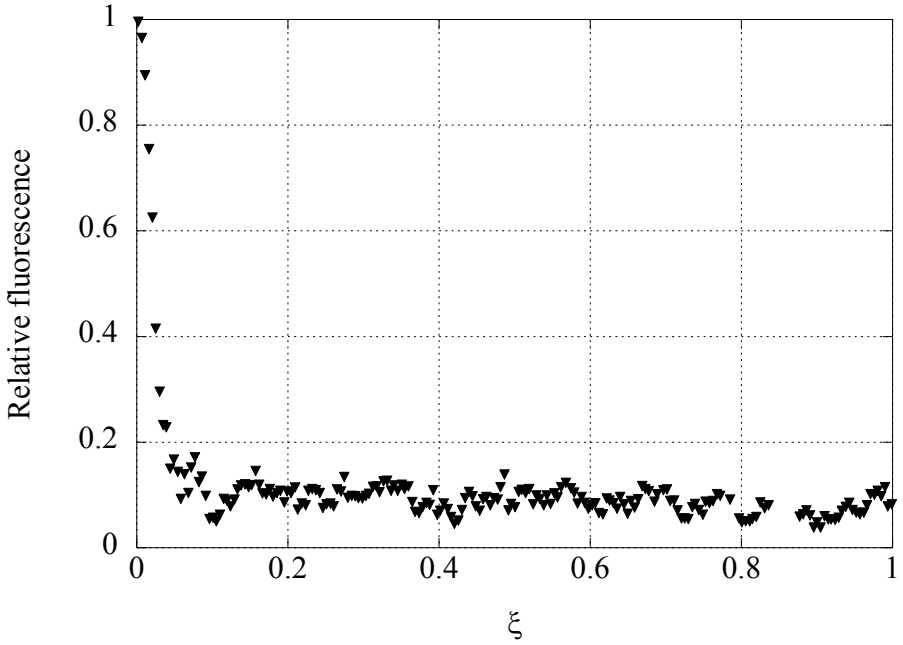

Figure 2: Concentration profile at 24 h of NE.

Discussion

Nanoemulsions have been successfully proposed to deliver active compounds on/into the skin. Among the different production techniques, the low-energy methods offer different advantages such as an easy production, as well as low equipment and operation costs (Anton N. *et al*., 2008). In this study, an isothermal low-energy method was used to spontaneously produce oil-in-water nanoemulsions for the topical administration of VK1. In particular, the formulative study was focused on the development of spontaneous nanoemulsions composed by α-TOC and K1 as oil phase, tween 80 as surfactant and water. In the first phase of the work, we carried out a formulative study to produce nanemulsions by spontaneous emulsification, in which we examined the influence of the ratio between the different component, mainly of the surfactant concentration, on the size of the NE droplets. The 10% w/w of tween 80 was found be the concentration suitable to give a good compromise between a stable emulsion with low size of the droples as well as with a narrow size distribution. More in detail, the increase of the surfactant-to-emulsion ratios (SER) from 1 to 20% led to a strong decrease of the droplet size, namely from large aggregates >1mm (undetectable in our experimental setting) to 100.5 nm. This phenomena could be attributed to an increased adsorption of surfactant molecules to the oil–water interface leading to a decrease in the interfacial tension, which facilitates the formation of smaller droplets as previously reported by other authors (Lamaallam *et al*., 2005). The polydispersity index did not follow the same trend of the mean diameter; in fact, value of PI decreased with increasing tween 80 concentration from 1 to 10%, while this value increased for further increase of the surfactant concentration. These results demonstrate the possibility to obtain NEs using the spontaneous emulsification method even at relatively low surfactant concentrations and at room temperature without the use of any high-energy equipment, which may be important for commercial development of these systems (Anton N. *et al*., 2008). Moreover, starting from the selected formulation, VK1-enriched NEs were developed; due to the high lipophilicity of VK1, the latter was added into the oil phase, replacing an equal amount of α-TOC, thus without changes in the total percentage of the oil phase. Thus, in the following step, VK1 was added in the NE at

different concentrations and its effects of the NE characteristics were investigated. Although the addition of VK1 led to an increase of the mean diameter of NEs, it was possible to obtain different NEs containing different amount of VK1 with a diameter of the droplets lower than 250 nm, a low PI and a negative ZP; moreover, all these NEs-VK1 were very stable during the storage at 4 ºC at different time frame (until 6 months).

Starting from the assumption that this work was focus on the development of a formulation to propose for commercial development, special attention was paid to the physical stability of the system to guarantee a long shelf-life. So the influence of storage time and the temperature on the stability of NEs-VK1 was examined. From the stability studies emerged that, although all NEs-VK1 were stable at the temperature of 4º C, little changes occurred in NEs-VK1 5% and NEs-VK1 1 % after storage at 25 and 40 º C. Only in the case of NEs-VK1 3%, the formulation showed a good stability also at room temperature with an increase of the droplets size of only 4% after 3 months and values of ZP and PI comparable during the time. From data obtained in this study, it is possible to conclude that VK1 concentration has only a weak effect of the NE stability. Moreover, the NEs were stable during the storage at 25 and 40°C, with only negligible changes of the NE technological characteristics. If we compare the stability of NEs at 25 and 40°C, with that obtained with liposomes, ethosomes and transfersomes (see previous chapters), it is possible to conclude that the phospholipids, used to prepared the vesicles, are reasonably responsible of the weak vesicle stability when increasing the storage temperature. Indeed, the use of phospholipid-free systems, e.g. NE, can allow to overcome the storage stability issues, thus providing formulation with a potential longer shelf-life. Ex vivo experiments also demonstrated the possibility to promote the VK1 accumulation into the skin by using NEs. Further details of these experiments have been omitted in the respect of a confidentiality agreement on this work.

Conclusions

In this chapter, VK1-containing NEs were designed and developed. The preparation protocol was designed to easily and economically prepare nanoemulsions avoiding the use of high-energy methods. The developed NEs had good proprieties in term of size of the droplets, stability over the time at different storage temperatures. The NEs investigated in this work could be proposed as the best option for the commercial development of a liquid and aqueous formulation for topical delivery of VK1. This is mainly due to the highest stability of the system. The further strength of this formulation is the absence of high energy agitation or extrusion step associated to the absence of expensive excipients (i.e. phospholipids), that make it certainly more suitable for a large scale cost-effective production.

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