Abstract:
The invention relates to vesicles for topical delivery of drugs and cosmetics, named glyceromes and characterized by high content of glycerol. The invention further relates to pharmaceutical formulations and cosmetic products containing glyceromes.
GLYCEROSQMES AND USE THEREOF IN PHARMACEUTICAL AND
COSMETIC PREPARATIONS FOR TOPICAL APPLICATIONS

The present invention relates to vesicular nanostructures useful for the formulation of active pharmaceutical ingredients and cosmetic products and to their application for topical delivery of drugs and cosmetics. The vesicular nanostructures described in the present invention are named glyceromes due to their high content of glycerol.

BACKGROUND OF THE INVENTION.

Topical drug delivery, compared to conventional routes of administration such as oral or parenteral delivery, is both potentially advantageous since it avoids active principle degradation in gastrointestinal tract and first pass hepatic metabolism and it is more acceptable by patients.

However the skin, which consists of two layers, the deeper one or dermis and the external layer or epidermis, behaves as a difficult to permeate barrier for most drug substances.

The deeper layer or dermis, whose thickness is between 0.3 and 4 mm, consists of connective tissue embedded with blood vessels, pilo-sebaceous units (hair follicles and sebaceous glands) as well as nerve endings which makes skin a true sense organ. At dermis level, active principles can cross the capillary walls to enter into the circulatory system and reach different tissues. The outermost layer of the skin or epidermis, whose thickness is between 50 and 150 µm, is covered by a hydrolipidic film and performs a barrier function against microorganisms and other exogenous molecules from the surrounding environment. Keratinocytes are the typical epidermis cells that originate at the innermost layer close to dermis and undergo a gradual differentiation process called keratinization ending with migration to surface to form a horny layer of dead cells (statum corneum) with thickness between 10 and 30 µm.
The horny layer acts as an effective barrier limiting the passage of active principles whose rate of transdermal absorption correlates with the generally very low rate of their penetration through the horny layer. Due to this barrier effect, the topical administration of drugs normally results in a reduced bioavailability.

Different approaches have been investigated to improve the diffusion of drugs through the skin including physicochemical methods based on the use of penetration enhancers such as dimethylsulphoxide, fatty acids, propylene glycol and urea [Williams AC e Barry BW, 1992] as well physical methods including, among others, iontophoresis, electroporation and low-frequency ultrasound [Lavon I e Kost J, 2004] or a combined application of both physical methods and chemical enhancers.

A different approach to improve the transdermal diffusion of drugs is based on the carrier properties of vesicular structures generally indicated as liposomes. Liposomes are microscopic vesicles of 50-500 nm containing amphipathic phospholipids which are molecules that have a polar head and a lipidic tail. In aqueous environment the hydrophilic heads line up to form a surface facing the water while the hydrophobic tails, repelled by water, line up to form a surface away from the water; moreover, the hydrophobic tails of one layer interact with the hydrophobic tails of another layer to form closed bilayer structures organized as uni-lamellar or multi-lamellar vesicles containing one or more aqueous compartments.

The chemicophysical properties of liposomes can be easily modified by variations of preparation methods and/or by including in their composition different substances such as, for example, cholesterol which increases the stability of lipidic bilayer, or acidic or basic lipid molecules which modify the electrical charge of vesicular surfaces and reduce liposome aggregation.

Hydrophilic compounds are solubilized in liposomes aqueous
compartments and hydrophobic compounds enter into the lipidic bilayers while amphipathic molecules are distributed with their polar portions in water and apolar portions in lipidic lamellae.

Moreover, ingredients used to prepare liposomes are safe, non-allergenic and being fully compatible with biological membranes enable the interaction of liposomes with cells.

Different mechanisms have been suggested to explain the interaction between liposomes and cells and some of them can possibly occur together. The most plausible mechanism is endocytosis where intact liposomes are phagocytated by cells and internalized into lysosomes where the drugs are released by phospholipase degradation of lipidic bilayers. Other putative mechanisms are based on lipidic substance exchange due to fusion of liposome bilayers with cell membranes followed by distribution of vesicle content between cytoplasmic compartment and cell membranes in dependence of its physicochemical properties.

The use of liposomes for targeted drug delivery at dermis and epidermis level with the aim to reduce systemic absorption and undesired effects has been first suggested in 1980 [Mezei M e Gulasekharam V, 1980; Mezei M, 1988] and then studied by many authors [Lichtenberg D e Barenholz Y, 1988; Woodle MC e Papahdjopoulou D, 1989, Sinico C et. al 2005, Manconon M et. Al 2006]. In some cases, liposomes can be used for transdermal permeation of drugs or cosmetic agents through skin appendages, such as hair follicles and sebaceous glands since, at this level, in dependence of liposome composition and preparation technique, active principles can cross the capillary walls and reach different tissues and organs from blood circulation [El Maghraby G.M et al., 2006]. In most cases, however, the use of liposomes as carriers for topical and/or transdermal administration of active principles is of little or no value since conventional liposomes do not penetrate the skin but rather remain...
confined to the upper layers of the stratum corneum. Later on, new classes of lipidic vesicles have been developed, namely deformable lipid vesicles and ethosomes, where the presence of specific additives modify the chemico-physical and functional properties of conventional liposomes enabling a more efficient delivery of drugs to deeper layers of the skin.

Deformable or flexible liposomes (also called Transferomes®) incorporate, besides the basal phospholipids component, single chain surfactants with a high radius of curvature (selected, for example, among sodium cholate, sodium deoxycholate, potassium glycyrrhizinate, Span 60, Span 65, Span 80, Tween 20, Tween 60, Tween 80) which are able to destabilizes vesicle lipidic bilayers and increase liposome deformability given them a higher skin penetration capability [Cevc G, Blume G, 1992].

Transferosomes are morphologically similar to standard liposomes even if they are functionally different being sufficiently deformable and flexible to penetrate pores much smaller than their own size. The mechanism of action of transferosomes is based both on a better capability of intact deformable vesicles to enter the stratum corneum and on subsequent modification and destabilization of intercellular lipidic structures which facilitate the diffusion of free drug molecules from the vesicles.

However, since the transport mechanisms of these deformable vesicles could partially depend on the physicochemical properties of incorporated drugs, the preparation of transferosomes need to be optimized on a case-by-case basis [Elsayed MMA et al., 2006].

A different approach to increase the fluidity of lipidic membranes of liposomes in order to improve their function as carriers of drugs and/or cosmetic products is represented by lipid vesicles called ethosomes composed of phospholipids, water and high concentrations, generally between 20% and 50%, of ethanol [Touitou E, US Patent 5,716,638]. High ethanol
concentrations reduce the ethosomes size and, in general, increase their encapsulation efficiency for a wide range of active principles including lipophilic molecules resulting in an effective delivery system for topical administration of drugs [Touitou E at al., 2000].

The action of ethosomes is possibly due to a synergistic mechanism between ethanol, lipid vesicles and skin lipids where the presence of ethanol could provide the vesicles with flexible characteristics for a better penetration into deeper layers of the skin as well as display its well-known permeation enhancing effect [Williams A, 2003].

Besides, ethosomes penetrated into the deep layers of the skin could fuse with skin lipids promoting the release of drugs at dermis level and possibly their transdermal absorption.

However, because of the high content of ethanol, the use of ethosomes as delivery system for drugs and/or cosmetic products may have unfavourable or irritanting effects in case of application on wounded or otherwise injured skin.

DESCRIPTION OF THE INVENTION

During the development of liposome-like formulations, the inventors have surprisingly found new vesicle structures with improved carrier characteristics for use in the pharmaceutical and cosmetic fields. These new formulations, called glycerosomes, are composed by phospholipids and water and are characterized by containing a high amount of glycerol (from 20 to 35%) which, unlike ethanol contained in ethosomes, is a harmless and fully accepted compound for topical applications. Additionally, unlike the liposomes known in the art [as for example in Fukui M, EP 1013268; Guilford FT, US 2006/0099244 and in Leigh S, WO 92/18103], the glycerosomes of the present invention are specifically characterized by being prepared in the absence of ethanol in order to avoid its irritating action following topical
application.

Glycerosomes according to the invention, whose glycerol content is between 20% and 35% w/w, preferably from 20% to 30%, more preferably 25%, can be prepared from any natural or synthetic phospholipids commonly used in the preparation of conventional liposomes known in the art; similarly, glycerosomes can contain one or more additives commonly used in the preparation of conventional liposomes known in the art and can be obtained by any of the different techniques commonly used for the preparation of conventional liposomes known in the art, e.g. according to the methods described in the following publications and patents herein incorporated by reference: Vemuri S, Rhodes CT. 1995; Cevc G. 2004; Torchilin VP, Weissig V, 2003.

In a further embodiment of the invention, glycerosomes can be used as carriers of active principles and incorporated in formulations for topical applications known in the art comprising, but not limited to, creams, emulsions, ointments, gels, lotions, suspensions and equivalent formulations.

Exemplary compositions of glycerosomes according to the present invention are reported in the following table 1:

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Glycerol</th>
<th>Modifiers</th>
<th>Absorption Enhancers</th>
<th>Active principles</th>
<th>Preservatives</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10%</td>
<td>20-35%</td>
<td>0-2%</td>
<td>0-5%</td>
<td>0-20%</td>
<td>0-1%</td>
<td>to 100%</td>
</tr>
</tbody>
</table>

The phospholipid components consist of one or more phospholipids extracted by natural sources or of phospholipids prepared by synthetic or semi-synthetic methods such as, for example, phosphatidylcholine (lecithin), phosphatidylethanolamine, phosphatidylyserine, phosphatidylglycerol, phosphatidylinositol, dimiristoylphosphatidylcholine (DMPC), dipalmitoyl
phosphatidylcholine (DPPC), distearoyl phosphatidylcholine, palmitoyl-
stearyl phosphatidylcholine, sphingomyelin, mixtures of soybean derived 
hydrogenated and non hydrogenated phospholipids such as Phospholipon 90 
(p90), Phospholipon 100 (pi00), Phospholipon 90G (p90G), Phospholipon
90H (p90H) as well as similar mixtures. The most relevant properties 
that the phospholipids should possess to be used in the preparation of glycerosomes
according to the invention are (i) capability to give vesicles which are able to
incorporate the active principles, (ii) biological compatibility, (iii) metabolic
degradation and (iv) lack of toxicity.

The modifier components, added to glycerosomes as stabilizers and/or
to modify their electric charge may be selected from cholesterol, stearylamine
and dicetylphosphate.

The absorption enhancers can be selected from dimethylsulphoxide, 
propylene glycol and fatty acids.

The glycerosomes of the present invention can be prepared according to
different approaches known in the art for preparing conventional liposomes
including, for example, the method of lipidic film hydration first described by
Bangham et al (1965) as well as methods based on the same principles [Kirby
C e Gregoriadis G, 1984]; the reverse-phase evaporation techniques [Skoza F,
Papahadjopoulos D, 1980; Papahadjopoulos D et al., U.S. Pat. No. 4,235,871;
Pick U, 1981]; the method of supercritical reverse-phase evaporation [Imura T
et al., 2002].

Depending on composition and method of preparation, the
glycerosomes of the present invention can results in unilamellar lipidic
vesicles (small or large) or in multilamellar lipid vesicles. Glycerosomes in
form of multilamellar vesicles can be converted in unilamellar vesicles by
sonication or homogenization, by extrusion or by filtration through
membranes with a specific pore size.
It is worth noting that glycerosomes containing more than 20% w/w glycerol are more effective than those having lower glycerol (for example from 10 to 15%) in several respects:

a) A higher glycerol (> 20%) concentration improve the rheological properties (e.g. viscosity) and, consequently, give a positive contribute to the stabilization of the glycerosome vesicular system.

b) A glycerol concentration lower than 20% hamper the assembling of vesicles at room temperature (namely at $25^0\text{C}$) when phospholipids with high transition temperatures (e.g. 70-80$^0\text{C}$) were used for preparing glycerosomes.

c) Vesicles prepared with a low glycerol concentration (10-15%) display a reduced flexibility and possibly a reduced skin permeation capacity.

Glycerosomes of the present invention can be used as carriers to deliver active principles of pharmaceutical or cosmetic interest including, but not limited to, lipophilic compounds such as acyclovir, trans-retinoic acid, betamethasone dipropionate, lidocain, minoxidil, amphotericin B, gentamycine, rifampicin, vitamin E and esters or lipophilic derivatives of vitamin E, etc. as well as hydrophilic compounds such as, for examples, diclofenac sodium, lidocaine hydrochloride, hyaluronic acid, vitamin C and its derivatives, soluble derivatives of vitamin E as vitamin E TPGS, etc.

To further illustrate the present invention, without limiting the field of application thereof, we have compared the chemicophysical and functional characteristics of glycerosomes with conventional liposomes - both prepared as described in Example 1 - as carriers for antiviral drug acyclovir, for highly lipophilic synthetic steroidal compound betamethasone dipropionate and for $\alpha$-tocopheryl polyethylene glycol-1000 succinate (Vitamin E TPGS or Tocophersolan) which is a water soluble form of natural vitamin E used in pharmaceutical field and as antioxidant in cosmetic preparations.
Acyclovir is an acyclic purine nucleoside analogue used for topical treatment of recurrent orofacial lesions, particularly in cases of herpes labialis, which are caused by type 1 Herpes simplex virus (HSV-I) infections; an acyclovir formulation for topical treatment is commercially available as a cream containing 5% of active principle under the brand name Zovirax.

As shown in Table 2, carrier vesicles containing the phospholipid dipalmitoylphosphatidylcholine (DPPC) and the modifier agent cholesterol were loaded with two concentrations of active principle, namely 5% acyclovir in preparations A and 1% acyclovir in preparations B.

Table 2. Percent composition of glycerosomes and liposomes loaded with acyclovir.

<table>
<thead>
<tr>
<th></th>
<th>DPPC</th>
<th>Cholesterol</th>
<th>Acyclovir</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerosomes – A</td>
<td>3</td>
<td>0.1</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Glycerosomes – B</td>
<td>3</td>
<td>0.1</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Liposomes – A</td>
<td>3</td>
<td>0.1</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Liposomes – B</td>
<td>3</td>
<td>0.1</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

The efficiency of active principle entrapment is calculated taking into consideration the amount non incorporated and separated during glycerosomes purification on a gel filtration column. In fact, the amount of active principle incorporated into purified glycerosome preparations is measured after disruption of purified vesicles by action of Triton-X100 or methanol. The efficiency of incorporation (E%) is then expressed as percentage of active principle taken up by vesicles with respect to the initial loading amount, according to the following equation:

\[ E\% = \frac{\text{amount of active principle of purified preparation}}{\text{initial load of active principle}} \times 100 \]
Glyceromes showed a significantly higher incorporation efficiency of acyclovir with respect to the incorporation in conventional liposomes, as reported in table 3.

Table 3. Incorporation efficiency of acyclovir in glycerosomes and liposomes.

<table>
<thead>
<tr>
<th>Incorporation efficiency of acyclovir (%)</th>
<th>Glycerosomes-A</th>
<th>Liposomes-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>54.4 ± 8</td>
<td>30.6 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

A further advantage of glycerosomes is represented by the fact that they are prepared at room temperature, more preferably at temperature lower than 30°C or, in a preferred embodiment at 25°C, irrespective of the type of phospholipid used for the preparation; on the contrary the operational temperature for preparing conventional liposomes corresponds to or is higher than the transition temperature (Tc) of phospholipids components which, in some cases, can be in the range of 60-80°C, as for example with DPPC with a Tc = 70°C. Avoiding any heating of the glycerosomes preparation mixtures represents an obvious advantage when the incorporated active principle is thermolable or can be partially decomposed upon heating.

Morphological analyses of acyclovir loaded glycerosomes by optical microscopy in polarized light enabled the identification of maltese crosses which confirm the presence of lamellar systems assimilable to vesicular structures [Manosroi A et al., 2003; Mele S et al., 2003; Sinico C et al 2005]. The presence of lamellar vesicular structures was confirmed by transmission electronic microscopy (TEM) analysis of glycerosome preparations, as shown in figure 1.

The stability of preparations was studied by dimensional analysis
according to the Photon Correlation Spectroscopy technique, by Z potential
determination, by Dynamic Laser Light Scattering and by measuring the polidispersity index at various time (up to 3 weeks) following vesicles
preparation.

Average size of glycerosomes and liposomes (as calculated in triple on
three different preparations) were respectively around 100 nm and 150 nm,
while the calculation of polidispersity index (PI), which represents an
important parameter to evaluate the homogeneity of vesicular preparations,
demonstrated higher homogeneity for glycerosomes with average PI values of
0.2 in comparison to average PI value of 0.4 for liposomes, as shown in figure
2. The better homogeneity of glycerosomes is a particularly useful property
for their use in delivery systems of pharmaceutical and cosmetic interest.

Besides, the high content of glycerol resulted in a higher glycerosomes
flexibility in comparison to conventional liposomes, as demonstrated by
measuring the mean particle size and the polydispersity index before and after
extrusion of the preparations through 50 nm pore size filters, as shown in table 4.

Table 4. Extrusion test of 5% acyclovir loaded glicerosomes and liposomes on 50 nm pore size filters.

<table>
<thead>
<tr>
<th></th>
<th>Before extrusion</th>
<th>After extrusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size</td>
<td>PI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glicerosomes -A</td>
<td>108 ± 33</td>
<td>0.23</td>
</tr>
<tr>
<td>Liposomes-A</td>
<td>148 ± 28</td>
<td>0.45</td>
</tr>
</tbody>
</table>

The results of table 4 clearly show the higher stiffness of conventional
liposomes which after extrusion showed a significant size reduction while polidispersity index increased due to the fact that only the vesicles whose size were comparable to the membrane porosity were filtered intact while larger vesicles were damaged and partially disrupted.

The higher glycerosomes flexibility represents a further useful property for their application as carriers for topical drug delivery since the use of more flexible vesicles can potentiate the penetration and transport of active principles into deep skin layers [Cevc G, Gebauer D., 2003].

Transdermal absorption from 5% acyclovir loaded glycerosomes and liposomes, that is the releasing and permeation of active principle through the skin, was assessed in a vertical Franz cell which is a suitable experimental approach to study percutaneous absorption of topical formulations using a membrane from newborn pig skin. As described in example 6, the cell was maintained in a thermostatic bath at skin physiological temperature of 32°C up to 24 hours; at the end of the experiment porcine skin specimen used in Franz cell as membranes were manually sectioned and stratum corneum, epidermis and dermis were separately analyze to measure the distribution of active principle in different skin layers.

In these experiments, 5% acyclovir loaded glycerosomes and liposomes were compared to a commercially available acyclovir cream for topical applications (Zovirax®, 5% acyclovir cream) and the results in terms of time dependent acyclovir permeation and acyclovir distribution in different skin layers are respectively shown in figure 3 and table 5.

According to the results of figure 3, 5% acyclovir loaded glycerosomes-B containing 25% glycerol showed a higher in vitro permeation flow (measured as µg acyclovir/cm² skin) when compared to both conventional liposome formulations and commercial 5% acyclovir cream. In particular, acyclovir permeation in 24 hours from glycerosomes was about 4 fold higher than
permeation from commercial cream formulation. The results of figure 3, showing that 1% acyclovir loaded glycerosomes-B gave a total permeation flow still 2 fold higher in comparison to commercial cream formulation can be considered a potential advantage in terms of safety enabling to use lower dosages and from economical/productive point of views enabling to reduce the amount of active principle.

The distribution of acyclovir among different cutaneous compartments after 24 hour permeation, as showed in table 5, demonstrate higher epidermis accumulation in the case of vesicular formulations while in the case of commercial cream the major amount of active principle was confined in stratum corneum. Even if accumulation at dermis and epidermis level did not show statistically significant differences among different formulations, the results of table 5 confirm differences in flow rates and strengthen the penetration enhancer capability of glycerosomes.

Table 5. Accumulation in different skin layers from 5% acyclovir loaded glycerosomes and liposomes in comparison to a commercial 5% acyclovir cream (Zovirax®). The experiments have been performed for 24 hours, in a Franz cell equipped with a diffusion membrane (0.636 cm²) from newborn pig skin.

<table>
<thead>
<tr>
<th></th>
<th>Stratum corneum</th>
<th>Epidermis</th>
<th>Dermis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/g</td>
<td>Dose adsorbed %</td>
<td>µg/g</td>
</tr>
<tr>
<td>Glycerosomes-A</td>
<td>67 ± 4</td>
<td>0.27 ± 0.002</td>
<td>227 ± 13</td>
</tr>
<tr>
<td>Liposomes-A</td>
<td>76 ± 9</td>
<td>0.30 ± 0.003</td>
<td>178 ± 35</td>
</tr>
<tr>
<td>Zovirax</td>
<td>527 ± 35</td>
<td>2.10 ± 0.140</td>
<td>183 ± 71</td>
</tr>
</tbody>
</table>

Betamethasone dipropionate is a lipophilic synthetic corticosteroid which as ointment, lotion or cream formulations is used for topical treatment
of corticosteroid responsive dermatitis due to local anti-itching, anti-inflammatory and vasoconstrictive effects.

The incorporation efficiency of betamethasone dipropionate is higher for glycerosomes than for conventional liposomes which respectively showed an incorporation index value E% of 68% and 42%.

The distribution in different cutaneous compartments of 0.2% loaded glycerosomes-A containing 25% glycerol and conventional liposomes was studied in 24 hour diffusion experiments using newborn pig skin in Franz cells; the results shown in figure 4 demonstrate that glycerosomes loaded with a highly lipophilic compound as betamethasone dipropionate increased the accumulation of active principle in deep skin layers.

D-alpha-tocopheryl polyethylene glycol-1000 succinate (Vitamin E-TPGS or Tocophersolan) is a water soluble derivative of natural vitamin-E prepared by esterification of free carboxylic group of vitamin-E succinate with 1,000 Dalton polyethylene glycol. Vitamin E-TPGS can enter in pharmaceutical formulations as emulsifier, surfactant and absorption enhancer and can be used in cosmetic formulations and skin care products because of its antioxidant properties.

Due to its amphiphilic properties, vitamin E TPGS is entrapped into lipidic bilayers of liposomes.

In vitro diffusion of 10% vitamin E TPGS loaded glycerosomes-A containing 25% glycerol and conventional liposomes was studied in Franz cells equipped with separation membranes from newborn pig skin and the results are shown in figure 5. Both formulations gave permeation profiles indicating a mechanism of controlled release as demonstrated by the low amount of 24 hour permeates (10-12 milligrams corresponding to about 5% of initially loaded product) and by the fact that, in the same experimental conditions, 20-22% of vitamin E TPGS permeated from an aqueous solution in
a steep burst within the first 4 hours (figure 5).

In the same experiments, the analysis of Vitamin E TPGS content in different skin compartments, as shown in table 6, demonstrated a higher accumulation in deep skin layers (deep epidermis/dermis) starting from 10% Vitamin E TPGs loaded glycerosomes with respect both to conventional liposomes and water solution.

Table 6. Accumulation of Vitamin E TPGS in deep skin layers (deep epidermis/dermis)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Vitamin E TPGS mg/gr skin tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water solutions</td>
<td>1.05 ± 0.49</td>
</tr>
<tr>
<td>Liposomes-A</td>
<td>1.80 ± 0.42</td>
</tr>
<tr>
<td>Glycerosomes-A</td>
<td>3.09 ± 0.58</td>
</tr>
</tbody>
</table>

The relationships between permeation profiles and accumulation in different skin compartments demonstrate that vesicular structures function as a Vitamin E TPGS depot system; this effect is greater with Vitamin E TPGS loaded glycerosomes which give a larger accumulation in deep skin layers and a burst-free permeation profile.

The combination of burst effect during permeation and of minor skin accumulation observed in the case of Vitamin E TPGS water solutions can be considered as an indication of higher permeation capability and of likely systemic absorption.

In conclusion, the glycerosomes according to the present invention, in comparison to conventional liposomes and other conventional formulations for topical administration such as creams, lotions, ointments or solutions display better permeation profiles acting as fully biocompatible skin penetration.
enhancers and are therefore useful carriers for topical administration of pharmaceutical active principles and cosmetic products with high skin permeation capability and without risks of cutaneous irritation.

In fact, the glycerosomes described in the present invention, being characterized by a glycerol content between 20% and 35% do not contain any aggressive component, such as ethyl alcohol, which after topical application could cause irritation or burn in the presence of cutaneous lesion as in the case, for example, of herpes labialis or other types of skin damages.

Below, experimental examples are given with the purpose to illustrate some preferred embodiments, without limiting the field of application of the invention.

**DESCRIPTION OF THE FIGURES**

**Figure 1**: Imaging of 5% acyclovir loaded glycerosomes by transmission electronic microscopy (TEM) analysis.

**Figure 2**: Polidispersity index (PI) of 5% acyclovir loaded glycerosomes-A and liposomes-A measured at various time following vesicles preparation.

**Figure 3**: Permeation profiles of 5% acyclovir loaded glycerosomes-A, glycerosomes-B and liposomes in comparison to a commercial 5% acyclovir cream (Zovirax®). The experiments have been performed for 24 hours, in Franz cells equipped with separation membranes (0.636 cm²) from newborn pig skin maintained at the physiological temperature of 32°C.

**Figure 4**: Distribution of 0.2% betamethasone dipropionate loaded glycerosomes containing 25% glycerol and conventional liposomes, after 24 hour permeation in Franz cells equipped with separation membranes (0.636 cm²) from newborn pig skin.

**Figure 5**: Permeation profiles of 10% Vitamin E TPGS loaded glycerosomes-A containing 25% glycerol and conventional liposomes in
comparison to Vitamin E TPGS aqueous solution. The experiments were performed for 24 hours in Franz cells equipped with separation membranes (0.636 cm\(^2\)) from newborn pig skin.

**Example N° 1. Preparation of glycerosomes and liposomes**

Glycerosomes and liposomes were prepared according to the lipidic film hydration technique. Briefly, weighted lipidic components (phospholipids, cholesterol, hydrophobic active principles or ionic lipids such as DCP or stearylamine) were dissolved in 5 ml of organic solvent (chloroform or dichloromethane) using a 50 ml round bottom flask containing 4-5 ml of 1 mm diameter glass beads. Solvent was removed using a rotary evaporator under vacuum at room temperature for 2 hours to get on the wall of the flask a lipidic film free of any trace of solvent. 5 ml of glycerol aqueous solution (glycerol concentration between 20% and 35%) also containing, if required, water soluble active principles were then added and the mixture was maintained under mechanical agitation at 10,000 rpm for 1 hour at 25°C using a crescent-shaped plastic stirring shaft in close contact with the internal round bottom flask surface. The emulsion, consisting of multilamellar vesicles, was maintained at rest for 1 hour and then homogenized at high-pressure for 10 minutes at 25°C using a Avestin Emulsiflex C5 homogenizer set at 60,000-65,000 kPa. Homogenated vesicles were purified by gel filtration on a Sephadex-G25 column to separate glyceromes, which were eluted in void volume, from non incorporated active principle. Column elution was carried out with water and the collected fractions were checked by UV turbidimetric analysis at 600 nm to identify the vesicle rich fractions.

Conventional liposomes were prepared as described but performing lipidic film hydration with aqueous buffer (for example with saline phosphate buffer); besides, both hydration and next homogenization phases were carried out at a temperature corresponding to or higher than the transition temperature.
(Tc) of the phospholipidic component (for example DPPC with Tc = 70°C; DMPC with Tc = 25°C; etc).

Example N° 2. Glycerosomes characterization.

Glycerosomes preparations were characterized by Transmission Electron Microscopy (TEM), optical microscopy in polarized light (PLM), Photon Correlation Spectroscopy (PCS) and by measurement of incorporation efficiency (E%).

For TEM, a drop of vesicular dispersion was applied to a copper grid, stained with 1% phosphotungstic acid solution and examined to confirm the vesicle formation as well as their morphology.

Vesicle dimensions and Z potential measurements were performed by a dynamic laser light scattering (DLLS) equipped with a Z-potential detector (Malvern).

For the calculation of E% values, corresponding to the fraction of drug incorporated into vesicles with respect to the initial loading (100%), loaded vesicles were separated from non incorporated drug by gel filtration on a column of Sepadex-G25 eluted with water.

Example N° 3. Quantitative analysis of acyclovir

Acyclovir quantitative determinations were performed by UV spectrophotometry according to the method described in British Pharmacopeia. Briefly, acyclovir containing samples were completely dissolved in 0.5 M sulphuric acid an lipophilic components were extracted with ethyl acetate and analyzed at a wavelength of 254 nm in comparison to a calibration curve.

Example N° 4. Preparation of porcine skin

In vitro permeation experiments were performed using newborn pig skin specimen obtained from local abattoirs, excised immediately after the animals' death, washed under running water, flatted on aluminum foils and
stored at -20°C.

Newborn pig skin is thin, characterized by a regular thickness and free of fats under dermal layer.

Example N° 5. Flexibility assay

Vesicle flexibility was evaluated by an extrusion test consisting in the measurement of average dimensions and polidispersity index before and after vesicle filtration through a 50 nm pore size filtering membranes.

Example N° 6. Permeation and accumulation studies

In vitro permeation studies were carried out in Franz diffusion cells under agitation with 500 µl vesicle samples loaded in upper cell compartment, or donor compartment. Specimen of frozen pig skin were rehydrated for 4 hours at room temperature in pH 7.4 phosphate buffer, cut in small discs and firmly secured between the two compartments of Franz cell by metal clamps. The available filtration surface of each disc was 0.636 cm². At consecutive time points during the experiments (0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours) the content of lower compartment was completely withdrawn and replaced with fresh buffer avoiding the formation of air bubbles under the skin. Collected samples were stored at 4°C pending the determination of active principle content.

At the end of the experiments, skin discs were air-dried. The horny layer was pulled off by applying to skin, for 10 seconds, a Tesafilm adhesive tape (type 40021) under light pressure and repeating the stripping after 40° rotation of skin specimen. Dermis, separated from epidermis with a surgical scalpel, was then cut in small pieces and homogenized in buffer solution by sonication; the suspension was filtered and quantitative active principle analysis was performed on filtered solution.

Each experiment was carried out in triplicate in three parallel cells and repeated at least three times.
REFERENCES


Fukui M. Compositions containing liposomes and/or emulsions and process for the preparation thereof. EP 1013268.


Lavon I, Kost J. Ultrasound and transdermal drug delivery. Drug


Touitou E. Composition for applying active substances to or through the skin. US Patent 5,716,638.


CLAIMS

1. Liposome-like vesicles for pharmaceutical and cosmetic topical applications (glycerosomes), comprising:
   a) from 20% to 35% w/w glycerol;
   b) from 1% to 10% w/w of at least one phospholipidic component;
   c) at least one pharmaceutically or cosmetically active principle.

2. Glycerosomes according to claim 1, containing from 20% to 30%, preferably 25% w/w glycerol.

3. Glycerosomes according to claim 1, wherein said phospholipidic component is selected from phosphatidylcholine (lecithin), phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, dimiristoylphosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine, palmitoyl-stearoyl phosphatidylcholine, sphingomyelin, mixtures of soybean derived hydrogenated and non hydrogenated phospholipids.

4. Glycerosomes according to claims 1 to 3, further containing a modifier of phospholipic membranes in concentration from 0 to 2% w/w and/or an enhancer of cutaneous absorption in concentration from 0 to 5% w/w.

5. Glycerosomes according to claims 1-4, wherein said modifier of phospholipid membranes is selected from cholesterol, stearylamine, dicetylphosphate and phosphatidylglycerol (PG).

6. Glycerosomes according to claims 1-5, wherein said enhancer of cutaneous absorption is selected from propylene glycol, menthol and fatty acids.

7. Glycerosomes according to claims 1-6, wherein the pharmaceutical or cosmetic active ingredient is selected from antiviral drugs, vitamins and derived thereof, vegetal extracts, antinflammatory drugs, corticosteroids,
antibiotics, fungicidal drugs, antipsoriasis drugs, acyclovir, trans-retinoic acid, betamethason and derivatives, lidocaine, minoxidil, amphotericin B, gentamycine, rifampicin, vitamin E and esters or lipophilic derivatives of vitamin E, diclofenac sodium, lidocaine hydrochloride, alkaloids, hydroxyacids, hyaluronic acid, vitamin C and its derivatives, soluble derivatives of vitamin E, vitamin E TPGS, antiwrinkles and antiaging compounds, antioxidants, products for hair treatments, humectants and sunscreen ingredients.

8. A process for the preparation of glycerosomes according to claims 1-7, comprising mixing 1-10% w/w phospholipids, 20-35% glycerol, 0.1-10% active principle, 0-2% phospholipid membrane modifier; 0-5% cutaneous absorption enhancer and water to 100%, until a colloidal system comprising unilamellar or multilamellar vesicles is obtained.

9. A process according to claim 8, which is carried out at room temperature, preferably at a temperature lower than 30°C, more preferably at a temperature of 25°C.

10. Use of glycerosomes according to claims 1-9 for the preparation of a pharmaceutical or cosmetic composition for topical administration.

11. Use according to claim 10, wherein said pharmaceutical or cosmetic composition is in the form of a cream, emulsion, ointment, gel, lotion or suspension.
FIGURE 1
FIGURE 2

![Graph showing the polidispersity index over time for Liposomes A and Glycerosomes.](image)
FIGURE 3

[Graph showing data for Liposomes A, Glycerosomes A, Glycerosomes, and Zovirax® over time (h) and concentration (μg permeated/cm² of skin).]
FIGURE 4

![Graph showing comparison of liposomes and Glyceroosomes 25% in terms of μg/cm² across different skin layers. The layers include Stratum corneum, Epidermis, and Dermis. The graph indicates differences in concentration across these layers for each type of vesicle.]
**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K9/127
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC:

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WO 92/18103 A (PHARES PHARMA HOLLAND [NL]) 29 October 1992 (1992-10-29) page 12 - page 14; example 2</td>
<td>1-11</td>
</tr>
</tbody>
</table>

Special categories of cited documents:

- **A** document defining the general state of the art which is not considered to be of particular relevance
- **E** earlier document but published on or after the international filing date
- **I** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- **O** document referring to an oral disclosure, use, exhibition or other means
- **P** document published prior to the international filing date but later than the priority date claimed

T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X** document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**Y** document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

**&** document member of the same patent family

Date of the actual completion of the international search: 2 June 2010

Date of mailing of the international search report: 09/06/2010

Name and mailing address of the ISA:

European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040,
Fax (+31-70) 340-3016

Authorized officer:

Mul l er, Sophie
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DE 69229969 T2</td>
<td>30-12-1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0724430 A1</td>
<td>07-08-1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2003044454 A1</td>
<td>06-03-2003</td>
</tr>
<tr>
<td>US 2006099244 A1</td>
<td>11-05-2006</td>
<td>NONE</td>
<td></td>
</tr>
</tbody>
</table>