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(54) Title: A METHOD OF FORMING A LIPID MEMBRANE COMPRISING STACKED LIPID LAYERS AS WELL AS A SUPPORT PROVIDED WITH A LIPID MEMBRANE COMPRISING SUC STACKED LIPID LAYERS, AND THE USE THEREOF

(57) Abstract: The present invention relates to a method of forming a membrane comprising stacked lipid layers on a support. According to the present invention, the lipid layers are applied on the support by means of spraying, after which a heat treatment is performed in absence of contact with an aqueous solution. The present invention allows forming a more homogeneous lipid membrane (formed by stacked lipid layers of lamellae) on the support. The invention also relates to a support provided with a lipid membrane, which in essence is free of holes, as well as applications for such a membrane.



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A method of forming a lipid membrane comprising stacked lipid layers, as well as a support provided with a lipid membrane comprising such stacked lipid layers, and the use thereof

5 The present invention relates to a method of forming a lipid membrane comprising stacked lipid layers using a lipid mixture containing at least 1 ceramide and capable of forming a stack of lipid layers, said method comprising the steps of

a) dissolving lipids in a solvent to yield a solution containing a
10 lipid mixture,

b) applying the solution containing the lipid mixture to a support,

c) allowing the solvent to evaporate in order to yield a support provided with a lipid membrane,

d) subjecting the support provided with a lipid membrane to a heat
15 treatment to yield a support having a lipid membrane enriched in structured lipid layers.

In the areas of pharmaceuticals and cosmetics it is very important to have at one's disposal a system capable of functioning as an in vitro model system for the skin, in particular the skin of mammals, more in particular that of humans. Such a model system allows
20 the simulation of the barrier properties of the skin. This offers several advantages, both with respect to reduction of the use of laboratory animals and to better reproducibility of experiments. After all, the properties of the skin differ depending on the location
25 on the body, and between individual mammals. In addition, with a model system the barrier properties can be changed purposively by choosing another lipid composition. This allows for providing model systems that are representative for the skin of people who have a diseased or dry skin.

30 The main function of the skin is impede the penetration of undesired compounds into an organism, such as a human being, via the skin. The most important barrier is provided by the outermost layer of skin which is referred to as the stratum corneum. This outermost layer of skin is characterized by cells (corneocytes) that are embedded in a matrix of stacked lipid layers. These lipid layers have a
35 unique composition as well as a unique structure. They contain, apart from lipids that are present in the body more generally, such as cholesterol, special free fatty acids having long chains and ceramides.

Each ceramide molecule comprises two long hydrocarbon chains, usually of different length. The first chain is referred to as an acyl chain. The second chain is referred to as a non-fatty acid chain, and is part of the sphingoid basic structure.

5 In Biochimica et Biophysica Acta 1372, p. 135-140 (1998) Kuempel et al disclose a method of forming a lipid membrane on a support, said lipid membrane consisting of stacked lipid layers (lamellae). As a possible use, in vitro investigation of transdermal drug delivery is mentioned. Kuempel et al disclose a method for the preparation of
10 lipid layers having the best possible imitation of (healthy) skin desired for long periodicity of about 13 nm. In short, the known method comprises the application of a solution containing a lipid mixture on a disc-shaped support by repeatedly pipetting. Between the individual applications the solvent is left to evaporate, yielding a lipid membrane. In order to form a lipid membrane composed of lipid layers
15 having the desired long periodicity, a heat treatment is performed. During this heat treatment the supports, with on top of them the lipid membrane, are hydrated by hanging them in distilled water and heating them at 80°C. After cooling, the disc-shaped support is
20 dried, whereby which the long periodicity in the lipid membrane is formed.

A disadvantage of the known method is that the lipid layers thus formed may contain inhomogeneities, which may be visible as holes with electron microscopy, as a result of which they are useless
25 for the intended purpose of permeability research.

The objective of the present invention is to provide a method according to the preamble, wherein said disadvantage is largely or even completely eliminated.

To this end, the present invention is characterized in that
30 i) the solution containing lipids of step b) is applied by means of a spraying method, such that the droplets formed using the spraying method do reach the substrate as droplets
ii) the heat treatment of step d) comprises heating to a temperature below the melting point or melting range of the lipid mixture on the
35 substrate, whereupon the lipid mixture thus heated is cooled in the absence of contact with an aqueous solution by at least 10°C, yielding the lipid membrane comprising stacked lipid layers.

Surprisingly, it has been found that the method according to the present invention yields a homogeneous lipid membrane comprising stacked lipids layers having no or virtually no holes and which, consequently, is useful for diffusion or penetration-experiments.

5 The present invention is not limited to a specific lipids composition.

According to a preferred embodiment the lipid mixture comprises at least 1 acyl ceramide capable of forming lipid layers having long periodicity or capable of enhancing the formation of such
10 lipid layers having long periodicity.

In the skin unique ceramide molecules are present, called acyl ceramides, said ceramides containing, apart from the above mentioned (first) acyl chain, a second acyl chain. This second acyl chain is present at the end of the first acyl chain that is not attached directly to the sphingoid basic structure; in other words, the second
15 acyl chain is linked indirectly to the sphingoid basic structure. It is believed that such acyl ceramide molecules which have an elongated acyl chain are essential for forming the unique structure of the lipid layers in the stratum corneum, as can be made visible with
20 electron microscopy and can be detected with X-ray diffraction. Only stacked lipid layers (lamellae) containing such acyl ceramides can display a unique long periodicity (namely, about 13 nm versus usually ca 6 nm). It is thought that within 1 lipid layer (lamella) that is part of a stack having long periodicity, the parallel (i.e., lying in
25 the plane of the lipid layer) areas of the lipid layer located at a distance of each other have a crystalline nature, whereas the part located in between these areas is of a more liquid nature. It is also believed that this specific structure contributes to the unique barrier properties of the skin. This results in a lipid membrane comprising stacked lipid layers having a long periodicity (in any case
30 >8 nm, usually ca 13 nm instead of ca 5 nm), these lipid membranes being extremely suitable for diffusion or penetration experiments because of their structure and their virtual absence of inhomogeneity.

It goes without saying that the lipid composition will have
35 to be chosen such that it is suitable for providing stacked lipid layers having a long periodicity. The ordinary person skilled in the art may, if necessary, start with the composition disclosed in the example and change this composition in one or more intermediate steps

until a composition desired by that ordinary person skilled in the art is reached. As is known in the art, several parameters play a role in the artificial formation of stacked lipid layers, such as temperature and the rate of evaporation of the solvent. In the event that during an intermediate step the quality of a stacked lipid layer deteriorates too much, these parameters may be varied to achieve a better quality of the lipid membrane. Subsequently, the next intermediate step can be performed, or the final desired composition may be used. It goes without saying that a lipids composition which resembles to a larger extent that which is known of the skin, will also display a behaviour more similar to the barrier properties of the skin to be simulated. Without being bound to any particular theory, it is believed that the chains of the acyl ceramide must have chain lengths differing such that 1 chain thereof must be so much longer than the chain length of the majority of other lipids, that this long chain can be part of two adjacent lipid layers. Although the invention is not limited to specific acyl ceramides, at least those acyl ceramides are included of which the first acyl chain has a backbone length of for example 20-42, such as advantageously 28 to 40 carbon atoms, preferably 30 to 32 carbon atoms. The second acyl chain linked to the first acyl chain has a backbone length of 12 to 25 carbon atoms, preferably 16-22 carbon atoms. The second chain of the acyl ceramide has a backbone length of 16 to 20 carbon atoms. The basic structure of the acyl ceramide may be sphingosine, phytosphingosine or 6-hydroxysphingosine. In the present invention a spraying method is understood to be any method of applying droplets to a support, such that the droplets are smaller than the distance between a nozzle from which a solution is sprayed and the support on which the droplets are sprayed from the nozzle. The droplets are, after coming into contact with the surface of the support, smaller than the surface of the support being covered with lipids. Usually there will be many droplets present between the nozzle and the support during spraying, which move in the direction of the support. The spraying method may be any known spraying method that does not cause the lipid components of the solution containing lipid mixture to be applied to the support in an unacceptable way, such as a piezo-electric spraying method. Without desire to be bound to any particular theory, it is believed that, based on the insight that some lipid components of the

solution are better soluble than others, transport of lipids over the surface of the support on which lipids are applied can be limited by means of a spray. It is believed that in this way a more homogeneous lipid membrane may be formed (in particular in the plane of the support). When in the context of the present invention heat treatment is mentioned, this includes the possibility of keeping the support at an elevated temperature during application of the solution, that is, at a temperature above room temperature (25°C). This enhances the elimination of the solvent through evaporation. The heat treatment can already be performed by heating during the application (that is, during step c)). The heat treatment depends on the lipid composition chosen, and comprises heating to at least 40°C, preferably to at least 50°C. The temperature is advantageously increased to less than 20° below the lowest point of the melting range of that particular lipid mixture, preferably to less than 15°, and more preferably to less than 10°C.

According to a preferred embodiment, the solution containing the lipid mixture contains an unsaturated lipid, and evaporation of the solvent is performed under an inert atmosphere.

In this way, the unsaturated lipid can be protected against oxidation by oxygen from the atmosphere.

According to an important embodiment, the solution containing lipids is applied using an airbrush spraying method.

Such a spraying method makes use of a carrier gas, the carrier gas contributing to the evaporation of the solvent from the surface. With a suitable combination of an elevated temperature of the support and a sufficiently high carrier gas rate (the carrier gas not being saturated in solvent), it is believed that the present invention may provide for continuous application of lipid mixture on the support.

The airbrush method is particularly suitable when a lipid has to be protected against oxidation by oxygen from its surroundings. Hence, according to a preferred embodiment the airbrush spraying method is performed using nitrogen or a noble gas as an inert carrier gas.

In order to obtain a lipid membrane having the least possible inhomogeneity (in the plane of the lipid membrane), it is preferred that the lipid mixture heated in step d) is cooled without contact

with an aqueous solution by at least 15°C, and preferably by at least 20°C.

To promote the formation of a lipid membrane having the desired long periodicity, it is preferred that the lipid mixture applied to the substrate, apart from acyl ceramides, comprises non-acyl ceramides, such that the lipid mixture used contains 5 to 40 mol% acyl ceramide relative to the total of ceramides in the lipid mixture, and preferably 10 to 15 mol%.

For the same reason, it is also preferred that the lipid mixture applied to the substrate comprises cholesterol and a total of the ceramides in a molar ratio of 0,4 to 2.

According to an important embodiment, the lipid mixture is present in the solution in such a concentration, that the solution is saturated for at least 50%, preferably at least 65% and more preferably at least 75%.

To prevent clogging of the nozzle, and to decrease the likelihood of lipid material being already solid before it ends up on the support, it is advantageous to avoid a saturated solution. A suitable upper limit is, for example, 95%.

In order to ensure a proper elimination of solvent from the deposited lipid membrane, to prevent accumulation of solvent and to avoid any concomitant problems it is preferred, according to the invention, to deposit the lipid membrane in thinner layers. Thus, one applies, for example, 25 to 500 thin layers for forming a lipid membrane having a total thickness of 15 µm. Hence, a preferred embodiment is characterized in that the lipid mixture is applied in such a quantity that the lipid mixture is applied repeatedly with a layer thickness each time, after drying, of 500 nm or less, preferably 250 nm or less, and more preferably less than 200 nm, such as less than 100 nm, where after each application the solvent is evaporated before the next layer of the lipid mixture is applied.

Although a lipid membrane can be applied on substantially any support by means of the method according to the invention, including for example a medical instrument, such as a tool as well as a utensil such as a catheter, that may come into in contact with a patient, it is preferred for an important application mentioned above - that is, a model system for the skin - that a macro porous support (membrane) is used as the support. Preferably, the pore size of the support is

in the range of 10 to 1000 nm, more preferably 25 to 200 nm. Such a membrane (macro porous support) provided with a lipid membrane using the method according to the invention is suitable for model experiments where such membranes provided with a lipid membrane are used instead of stratum corneum obtained from laboratory animals or a human being.

Thus the present invention also relates to a support provided with a lipid membrane applied by means of the method according to the invention.

The lipid membrane on the support preferably has a size of at least 0.1 mm², a size for lipid membranes with few defects not achievable without the method according to the invention. In this context, with few defects means that any holes present increase the flux of diffusion through the membrane by at most 50%.

Preferably the support is a membrane (in the sense of a macro porous support).

Such a membrane as a support has advantageously a pore size of 0.5 µm or less.

Finally, the invention relates to the use of a support according to the invention. More specifically it concerns a method of measuring the permeability of a lipid membrane, wherein a member chosen from a vaccine, a parasite, a drug, a bacterium, a fungus, a peptide, a protein, DNA, RNA, an allergen, a lipid, a solvent and a moisturiser is applied at one side of a support according to the invention, and the penetration through the support is measured.

The present invention will now be illustrated with reference to some examples and with reference to the drawing, wherein

fig. 1 diagrammatically represents an apparatus suitable for applying lipid layers on a support 1 using the method according to the invention;

fig. 2 shows a detail of the apparatus of fig. 1 to clarify the operation of the apparatus;

fig. 3a and b are scanning electron microscopy pictures of supports provided with a lipid membrane, wherein the supports have been subjected to a heat treatment in the presence and absence of an aqueous solution respectively;

fig. 4 is a picture of plates obtained using thin layer chromatography, the plates showing the distribution of lipids in the cen-

tre of the support (left) and at the periphery thereof (right) respectively, for a support prepared using the method according to the invention;

fig. 5 corresponds to fig. 4, except that it is for a support not prepared using the method according to the invention (control);

fig. 6a and 6b show an X-ray diffraction pattern of a lipid membrane prepared using the method according to the invention, and a graphical representation of the intensity (from left to right, through the centre of fig. 6a) respectively;

fig. 7 shows the results of diffusion experiments, using isolated humane stratum corneum as a control; and

fig. 8 depicts a graph of a diffusion experiment in the presence and absence of an acyl ceramide.

1. Materials

CER1(C30)-linoleate, CER2(C24), CER3(C24), CER3(C16), CER4(C24) and CER6(C24) were gifts from Cosmoferm B.V. (Delft, The Netherlands). Palmitinic acid, stearic acid, arachidinic acid, beheninic acid, tricosanonic acid, lignocericinic acid, cerotinic acid, and cholesterol were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). All organic solvents used were of analytical quality and made by Labscan Ltd (Dublin, Ireland). As a support (substrate) for the lipid membranes to be prepared, Nuclepore polycarbonate filter disks (pore size 50 nm) by Whatman (Kent, UK), were used. All other chemicals were of analytical quality and the water used was of MilliQ quality.

2. Airbrush set-up

For spraying the solution containing lipids onto the porous support, use was made of a spraying apparatus 1 as depicted in figure 1. Visible are a support frame 2 in which an Evolution solo airbrush 3 (Airbrush Service, Almere, The Netherlands) with a nozzle 4 (opening 0.6 mm) is mounted vertically, the nozzle 4 pointing downwardly. For the support frame 2 the frame of a microscope with a substrate table 5 is used, onto which a support A is clamped for the method according to the invention. By means of the focussing means 6 of the microscope, the distance between the nozzle 4 and the support A on the substrate table 5 can be set. In the present case, the distance was variable between 2.5 and 8.0 cm. The airbrush 3 is connected to a nitrogen bottle (not shown) via a rapid action coupling of type NW 2.7 mm. As

will be explained hereinafter, in the embodiment discussed below, use is made of two streams of nitrogen having a first high pressure and a second low pressure respectively. To this end, the apparatus has a first pressure reducing valve 7 to reduce the nitrogen pressure as present in the nitrogen bottle, to the first high pressure. Nitrogen at his first high pressure passes via conduit 8 to a three-way valve 9 (24 V DC 1.8 W, Kuhnke GmbH, Malente, Germany). To obtain the second low pressure, a second pressure reducing valve 10 is provided in a conduit 11 branched off from conduit 8 which is also connected to three-way valve 9. Depending on the position of the three-way valve 9, nitrogen is passed under a first high or second low pressure to the airbrush 3 to atomize the solution B respectively to dry the solution B sprayed on the support A. In the apparatus 1 described here, the pressure reducing valves 7 and 10 allow the desired pressure to be set between 0 to 1.6 bar and 0 to 400 mbar, respectively. The pressures used for spraying and drying can be read from the manometers 12 and 13 (tube spring manometer 1401A R40, Eriks BV, Alkmaar, The Netherlands).

The apparatus 1 used for applying a lipid membrane, using the method according to the invention makes it possible to control the duration and the flow rate at which solution B is sprayed. To this end, the trigger 14 of the airbrush 3, which also controls trigger 14, as is usual, the supply of liquid B in the airbrush 3 for atomization by the airbrush 3, is controlled pneumatically. To this end, a conduit 15 is connected to a source of compressed air (not shown) to supply compressed air G. To operate the trigger 14, the pressure of the compressed air G is reduced to 3 bar using a pressure reducing valve 16, whose pressure can be read from manometer 17. The trigger 14 of the airbrush is opened pneumatically by means of a standard pneumatic actuator unit 20. This unit comprises a rodlet 21 that is kept in the actuator unit 20 in a pushed-in position in the actuator unit 20 by a spring (not shown), and is brought to a pushed-out position by compressed air G. The supply of compressed air G is controlled by valve 23. In figure 1 the rodlet 21 is in a pushed-in position, in figure 2 it is in the pushed-out position. The distance over which the trigger 14 can be moved, and consequently the rate at which solution B can be atomized by the airbrush 3, can be varied with an adjusting screw 19. In the present case, the distance could

be set between 0 and 8 mm, whereby 0 mm was the distance at which the trigger 14 is just sufficient to prevent passage of solution B.

The solution containing lipids B is pipetted into the liquid tank 22 of the airbrush from where it is passed via a conduit (not shown) in the airbrush 3 to the nozzle 4, where the solution B is atomized.

Control of the three-way valve 9 and valve 23 is achieved via an electronic control panel (not shown).

In summary, the apparatus 1 described above allows the following parameters to be varied: the nitrogen pressure during atomization, the nitrogen pressure during drying, the distance between the nozzle and the support, the time during which the trigger is opened, the distance over which the trigger is opened, the time period between 2 consecutive sprays and the total number of sprays after one another and the temperature of the support.

3. Preparation of a lipid membrane on a support

a) The lipids shown in table 1 were dissolved in hexane-ethanol (2:1 vol./vol.) at the indicated molar ratio. The total concentration of lipids was 4.5 mg/ml (90% of the saturating concentration).

Table 1

component	molar ratio
Ceramid 1 (C30)-linoleate	0.150
Ceramid 2 (C24)	0.511
Ceramid 3 (C24)	0.162
Ceramid 4 (C24)	0.033
Ceramid 3 (C16)	0.091
Ceramid 6 (C24)	0.052
cholesterol	1
palmitic acid	0.013
stearic acid	0.033
Arachidinic acid	0.067
beheninic acid	0.417
tricosanonic acid	0.054
lignocericinic acid	0.368
cerotinic acid	0.047

b+c) To achieve good atomization of the solution containing lipids, the nitrogen pressure maintained high just previous to and during a spray. To ensure an even drying process and to prevent solution B being forced outwardly over the surface of the support A, the nitrogen pressure is reduced immediately after the spraying period until the solvent is evaporated completely. This is checked visually (disappearance of the glossy appearance of the material deposited). Subsequently the procedure is repeated until a desired layer thickness of the lipid membrane is achieved. The support A is clamped to the substrate table 5. This is necessary to ensure an even surface during spraying and in addition this prevents the support A from being blown away by the nitrogen pressure. The support is placed directly under the nozzle 4 of airbrush 3.

As a support a Nuclepore polycarbonate filter is mounted with its glossy side up in a filter holder and is placed on the substrate table 5 of the apparatus 1. The distance between the nozzle 4 and the filter, which has a pore size of 50 nm, is about 4.5 cm. Subsequently, the following parameters are set:

Nitrogen pressure high immediately before and during atomization:

1.16 bar (absolute).

Spraying duration: about 1 second.

Nitrogen pressure before drying (thus after atomization of solution B on the support): 160 mbar.

Drying duration after each time of spraying: about 15 seconds.

Distance over which the trigger is opened: 5.5 mm

Volume of the solution applied by spraying: 330 µl.

Number of sprays: 40.

To determine the (in)homogeneity of membranes prepared using the method according to the invention and a control, 330 µl of solution containing lipids was applied on a filter in 40 sprays as indicated above for electron microscopy and thin layer chromatography. For diffusion experiments, 750 µl was applied by spraying 90 times. The other settings were the same.

d) The filters provided with a lipid membrane are subsequently subjected to a heat treatment by heating them for 10 minutes at 70°C on an electrical hot plate. During this procedure, the filters are shielded from the ambient air by means of a glass plate placed above

the filters. After this 10 minutes of heating, the filters are removed from the heated plate and are cooled at ambient temperature, the glass plate remaining over the filters. In a control experiment, 10 ml 50 mM sodium acetate buffer (pH 5.0) was instead pipetted onto the filter after 10 minutes of heating at 70°C. Subsequently, everything as described above, covered with a glass plate, was brought to room temperature.

The filters with a lipid membrane were stored under argon in a refrigerator until further use.

4. Electron microscopy

The filters obtained from 3d) are cut to small pieces of about 1.5 x 2 mm², folded and mounted in Tissue-Tek O.C.T. Compound (Miles Inc. Elkhart, IN, US). The samples are subsequently frozen quickly by plunge-freezing (Reichert Jung-KF80, Vienna, Austria) in liquid propane at -180°C. Of the frozen samples, transsectional coupes were made using a cryo ultramicrotome (Leica Ultracut UCT/Leica EM FCS, Wetzlar, Germany), during which the temperature of the sample was -90°C and the temperature of the knife was -100°C. The filter pieces are dried for 3 min at -90°C and 0.1 Pa. Subsequently, a layer of platinum was applied by means of sputtering (CT 1500 HF, Oxford Instruments, UK). At least 5 pictures of each filter were taken at -190°C with a field emission scanning electron microscope (Jeol 6400F, Tokyo, Japan).

Result

Figure 3a and 3b show electron microscopic pictures of a lipid membrane which has been subjected to a heat treatment in the presence of sodium acetate buffer, and a lipid membrane obtained using the method according to the invention, respectively. In figures 3a and 3b the layer containing pores, on top in the lower half of each picture, is the support (filter), whereas the layer on top of that is the lipid membrane. At the outer top and bottom some Tissue-Tek mounting material is visible, which is of no relevance.

It can be seen that the lipid membrane of the control (fig. 3a) - where the cooling as part of the heat treatment is performed in the presence of the acetate buffer - is showing holes, evidence of inhomogeneities in the lipid membrane. Without wishing to be bound to any theory, it is believed that the inhomogeneous nature promotes pene-

tration by water and/or contributes to a phase separation, and that the holes visible in fig. 3a are the result of evaporation and expansion of water under reduced pressure. When, in accordance with the invention, cooling is performed in the absence of water, a layer without holes is obtained (fig. 3b). As is evident from the X-ray diffraction experiments, a lipid membrane having the desired long periodicity nonetheless is formed. Without wishing to be bound to any theory, it is believed that using the method according to the invention, where accumulation of a solvent on the lipid membrane and transport of the solution over the surface of the lipid membrane being formed is avoided, already initiates the desired structure with long periodicity.

The picture of the lipid membrane according to the invention (fig. 3b) reveals a small crack. It can also be seen that the lipid membrane has separated from the support. It is believed that these phenomena are artefacts caused during preparation of the lipid membrane concerned for electron microscopic investigation. The phenomena were not observed in other experiments.

5. Investigation of the homogeneity

To determine the homogeneity of the lipid membrane, thin layer chromatography (TLC) was performed. Filters obtained from 3d) were cut into two circular parts: the centre (diameter 4 mm; surface area 12.6 mm²) and the circumference (diameter 9 mm; surface area 51.0 mm²).

The lipids were extracted from the filter pieces by vigorous vortexing in 0.5 ml hexane:methanol (2:1). After evaporation the extracted lipids were dissolved in the same solvent in a volume corresponding to the surface area of the particular filter piece. Samples were placed under a stream of nitrogen with a Camag Linomat IV (CAMAG, Muttenz, Switzerland) on a silica plate (Merck, Darmstadt, Germany). In addition and as a control, different amounts of the original solution containing lipids were applied to the silica plate. By comparing the intensities of the various lipid bands originating from the lipid layers applied, with those of the solution containing lipids, the distribution of lipids on the filter can be determined. After elution with various organic solvent mixtures, the silica plate was sprayed with copper sulphate (Weerheim and Ponc, Arch. Dermatol. Res. 293, pp. 191 - 199 (2001)). After charring on a hot plate (10 min at 70°C,

followed by heating to 170°C, this temperature being maintained for 10 min), the intensities of the lipid bands were determined with a photodensitometer with automatic integration of peaks (Biorad GS 710, Hercules, CA, US).

5 Results

Figures 4 and 5 show silica plates developed with copper sulphate, obtained using thin layer chromatography. The left lane of each of 4 and 5 contains the lipids from the centre part, whereas the lane to the right (fig. 4 and 5) contains lipids from the periphery. The lipophilic nature of the lipids decreases from top to bottom: cholesterol, free fatty acids, ceramides 1 to 6. It can be seen that the distribution of the various lipids in the central part and peripheral part thereof is even for the lipid membrane obtained using the method according to the invention (fig. 4), as the intensities of the lipid bands between the left and right lane are comparable. In contrast, for the control experiment this distribution of lipids (fig. 5) is not homogeneous, the concentration of lipids being 2 mg.

In general, if the chromatogram of the outermost ring of the filter and that of the centre differ visually, it is recommended to take one or more of the following measures:

- use a more saturated lipid mixture solution,
- work discontinuously and let the solvent evaporate every time, or, if already working discontinuously, apply thinner layers per application.

It will be evident for the ordinary person skilled in the art in view of the teachings of the present invention that other measures may be considered to avoid redissolution of the deposited lipid membrane and to avoid transport parallel to the surface of the support, such as (moderately) increasing the temperature of the gas used for spraying, or to reduce the rate at which the solution containing lipids is sprayed (for example by reducing the distance over which the nozzle is opened). By means of routine experiments the ordinary person skilled in the art can easily determine suitable parameters for the lipid mixture chosen by this person skilled in the art.

35 6. X-ray diffraction investigation

X-ray diffraction investigations were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) using synchrotron radiation at station BM26B. The wavelength of the X-rays was 1.24 Å and

the distance between the sample and the detector was 1.7 meter. The diffraction data were collected at a 2-dimensional detector. The detector was calibrated with a sample of silver behenate having a thickness of about 1 mm. Tiny pieces of filter (dimensions about 2 by 10 mm) were mounted in a sample holder with mica windows. Measuring time per sample: 5 min.

Small angle X-ray scattering was used to obtain information on the orientation and lamellar organisation (the repeat distances of the lamellae). The scattering intensity I (in arbitrary units) was measured as a function of the scattering vector q (in nm^{-1}). The latter is defined as $q = (4\pi \sin \theta) / \lambda$, wherein θ is the scatter angle and λ is the wavelength. From the positions of a series of peaks located at equal distance of each other (q_n), the repetition distance d of a lamellar structure can be calculated, using the formula $q_n = 2n\pi/d$, where n represents the order of the diffraction peak ($n = 1, 2, 3, \dots$).

The diffraction pattern (fig. 6a) of the lipid membrane having the above mentioned composition shows five diffraction peaks ($q = 0.51, 1.02, 1.53, 2.04$ and 3.06 nm^{-1} ; fig. 6b. $I = \text{intensity}$) which can be attributed to a phase having a long periodicity and a repetition distance of 12.2 nm. The reflections at $q = 1.18, 2.36$ and 3.54 nm^{-1} correspond to the first, second and third order of a short periodicity phase having a repetition distance of 5.4 nm. The two reflections at 1.87 and 3.74 nm^{-1} (marked with an asterisk in fig. 6b) indicate that a small portion of the cholesterol crystallizes as separate domains. The diffraction pattern largely resembles that of the stratum corneum (not shown).

7. Investigation of the permeability

In order to determine whether the membranes formed according to the invention are suitable as a model for simulating the skin barrier, in vitro permeation investigations were performed using Permeagear inline diffusion-cells (Permeagear, Riegelsville, US) with a diffusion-surface area of 0.28 cm^2 . As a control, humane stratum corneum on a dialyses membrane (apical side facing the donor room) was used. Filters provided with a lipid membrane (after having, of course, been cooled according to the invention) were provided in the diffusion cell and prior to the experiment hydrated for 1 hour in phosphate

buffered saline of pH 7.4. The donor compartment was filled with 1280 μ l sodium acetate buffer of pH 5.0 in which a model compound was dissolved. The experiments were performed using three different model compounds: p-amino benzoic acid (PABA), ethyl-PABA and butyl-PABA.

5 The acetate buffer was saturated with the model compound in order to obtain equal and maximum thermodynamic activities. The acceptor compartment contained phosphate-buffered saline of pH 7.4, and this compartment was perfused at a flow rate of about 2 ml/u. The experiments were performed under occlusive conditions (that is: sealed off from
10 the surroundings), wherein the opening of the donor compartment was sealed off with a piece of adhesive tape. During the experiment, the temperature of the stratum corneum or that of the filter with the lipid membrane was kept at 32°C, using a thermostat controlled water bath. For a period of 20 hours, with 1 hour intervals, samples (2 ml)
15 from the acceptor compartment were collected in glass tubes. At the end of each experiment the steady state flux and the lag time were determined. This was done by plotting the cumulative amount of each of the model compounds that passed the stratum corneum and the lipid membranes respectively against time. The steady state flux was calculated using linear regression and is equal to the slope of the
20 straight part of the curve. The lag time equals the cut-off of the straight line at the X-axis (that is, the time).

Results

The results (Figure 7) show that the highest flux (J_{ss}) is obtained
25 with ethyl-PABA and that an increase (butyl-PABA) or decrease (PABA) in the lipophilic nature is accompanied by a significant decrease in the flux. This phenomenon is observed at the stratum corneum (SC) as well as at the lipid membranes (LM). The results further show that the lag time (t_{lag}) for PABA is significantly longer than that of the
30 two more lipophilic compounds ethyl-PABA and butyl-PABA. The lag times (t_{lag}) of the three model compounds and the steady state fluxes (J_{ss}) through the stratum corneum and the lipid membranes are similar. This means that the lipid membranes properly mimic the barrier properties of stratum corneum and that for this reason they are use-
35 ful for permeability measurements. Compared to stratum corneum the steady state flux of the most lipophilic compound butyl-PABA is somewhat elevated, whereas a shorter lag time is observed. For the most hydrophilic compound PABA however, the lag time is somewhat longer

than for stratum corneum. This indicates that the lipid layers are slightly more permeable for lipophilic compounds and slightly less permeable for hydrophilic compounds. This is explained by the fact that the membranes merely consist of lipids on a porous support, whereas the stratum corneum contains hydrophilic components as well. The observed differences in steady state flux between stratum corneum and the membrane, however, are very small compared to the differences observed with other skin barrier models, such as animal skin or artificially grown skin (results not shown). It is noted that other choices of the lipid composition may further reduce the differences.

8. Differences between membranes having short and long periodicity.

To study the effect of the composition of the lipid membranes according to the invention on the diffusion of ethyl-PABA, the above mentioned experiment was repeated, but ceramide 1 was left out of the composition (proportions of the other components of the lipid mixture were kept the same). The experiment was performed with three membranes for each of the two lipid mixtures. The experiment was performed twice for each lipid membrane. In each case, the lipid membrane had a thickness of 12 μm . The used diffusion surface area of the lipid membrane was 0.27 mm^2 .

Results

The lipid membranes prepared using the lipid mixture without ceramide 1 did not result in a structure having a long periodicity; the lipid membranes with ceramide 1 did (periodicity 13 nm). As can be seen in fig. 8, the membranes without a lamellar structure (-▲-) are twice as permeable as those (-□-) that do have the structure with long periodicity. In fig. 8, the time t is shown in hours on the X-axis and the permeability F ($\mu\text{g}/\text{cm}^2/\text{hour}$) on the Y-axis.

CLAIMS

1. A method of forming a lipid membrane comprising stacked lipid layers using a lipid mixture containing at least 1 ceramide and capable of forming a stack of lipid layers, said method comprising the steps of

a) dissolving lipids in a solvent to yield a solution containing a lipid mixture,

b) applying the solution containing the lipid mixture to a support,

c) allowing the solvent to evaporate in order to yield a support provided with a lipid membrane,

d) subjecting the support provided with a lipid membrane to a heat treatment to yield a support having a lipid membrane enriched in structured lipid layers,

wherein

i) the solution containing lipids of step b) is applied by means of a spraying method, such that the droplets formed using the spraying method do reach the substrate as droplets

ii) the heat treatment of step d) comprises heating to a temperature below the melting point or melting range of the lipid mixture on the substrate, whereupon the lipid mixture thus heated is cooled in the absence of contact with an aqueous solution by at least 10°C, yielding the lipid membrane comprising stacked lipid layers.

2. The method according to claim 1, wherein the lipid mixture comprises at least 1 acyl ceramide capable of forming lipid layers having long periodicity or capable of enhancing the formation of such lipid layers having long periodicity.

3. The method according to claim 1 of 2, wherein the solution containing the lipid mixture contains an unsaturated lipid, and evaporation of the solvent is performed under an inert atmosphere.

4. The method according to any of the preceding claims, wherein the solution containing lipids is applied using an airbrush spraying method.

5. The method according to claim 4, wherein the airbrush spraying method is performed using nitrogen or a noble gas as an inert carrier gas.

6. The method according to any of the preceding claims, wherein the lipid mixture heated in step d) is cooled in absence of contact with an aqueous solution by at least 15°C, and preferably by at least 20°C.

7. The method according to any of the preceding claims, wherein the lipid mixture applied to the substrate, apart from acyl ceramides, comprises non-acyl ceramides, such that the lipid mixture used contains 5 to 40 mol% acyl ceramide relative to the total of ceramides in the lipid mixture, and preferably 10 to 15 mol%.

8. The method according to claim 7, wherein the lipid mixture applied to the substrate comprises cholesterol and a total of ceramide in a molar ratio of 0.4 to 2.

9. The method according to any of the preceding claims, wherein the lipid mixture is present in the solution in such a concentration, that the solution is saturated for at least 50%, preferably at least 65% and more preferably at least 75%.

10. The method according to any of the preceding claims, wherein the lipid mixture is applied in such a quantity that the lipid mixture is applied repeatedly with a layer thickness every time, after drying, of 500 nm or less, preferably 250 nm or less, and more preferably less than 200 nm, such as less than 100 nm, where after each application the solvent is evaporated before the next layer of the lipid mixture is applied.

11. The method according to any of the preceding claims, wherein a macro porous support is used as the support.

12. The method according to claim 11, wherein the pore size of the support is in the range of 10 to 1000 nm, preferably 25 to 200 nm.

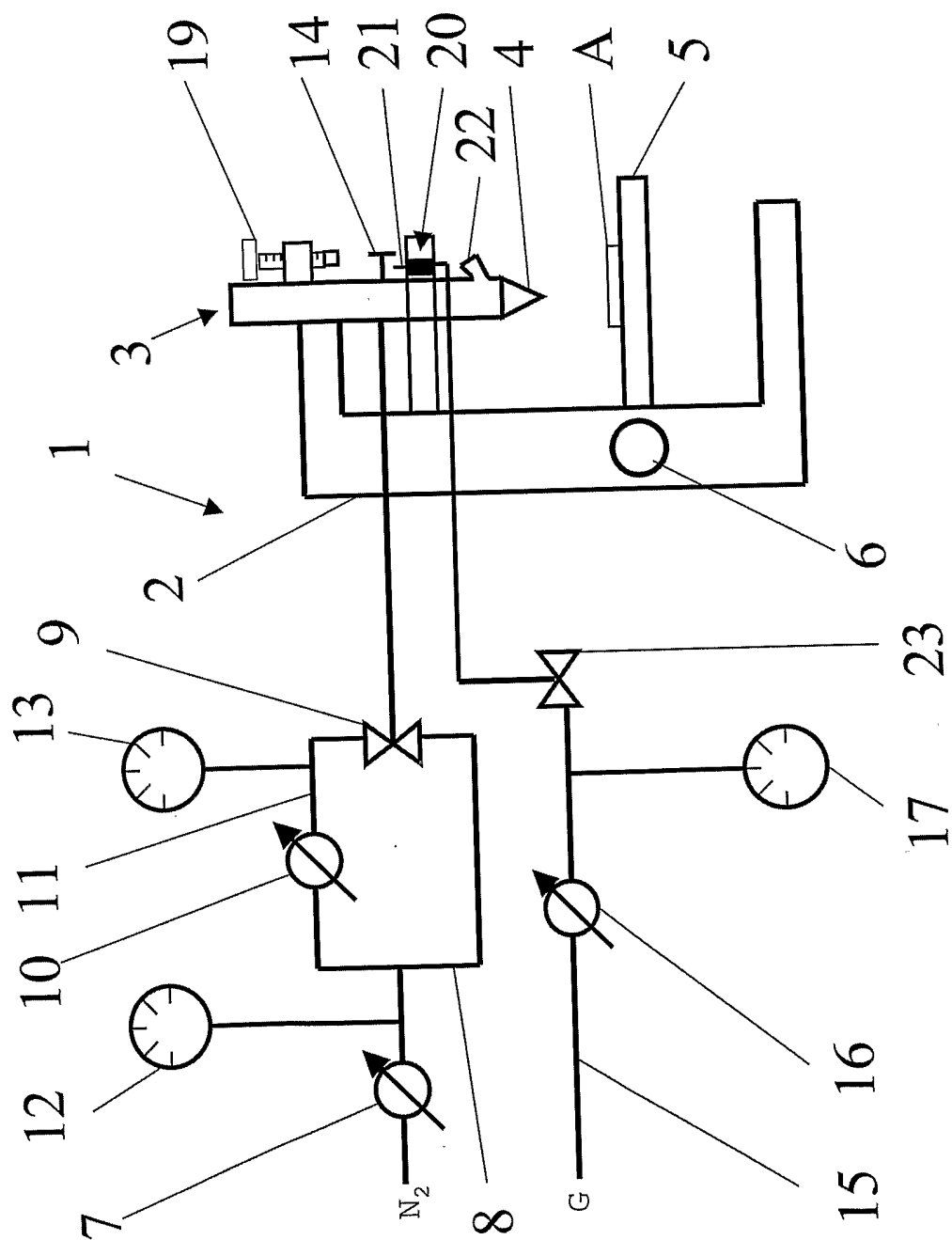
13. A support provided with a lipid membrane applied using the method according to any of the claims 1 to 11.

14. The support according to claim 13, wherein the support is a membrane.
5

15. A method of measuring the permeability of a lipid membrane, wherein a member chosen from a vaccine, a parasite, a drug, a bacterium, a fungus, a peptide, a protein, DNA, RNA, an allergen, a lipid,
10 a solvent and a moisturiser is applied at one side of a support according to claim 13 or 14, and the penetration through the support is measured.

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Fig. 1



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Fig. 3a

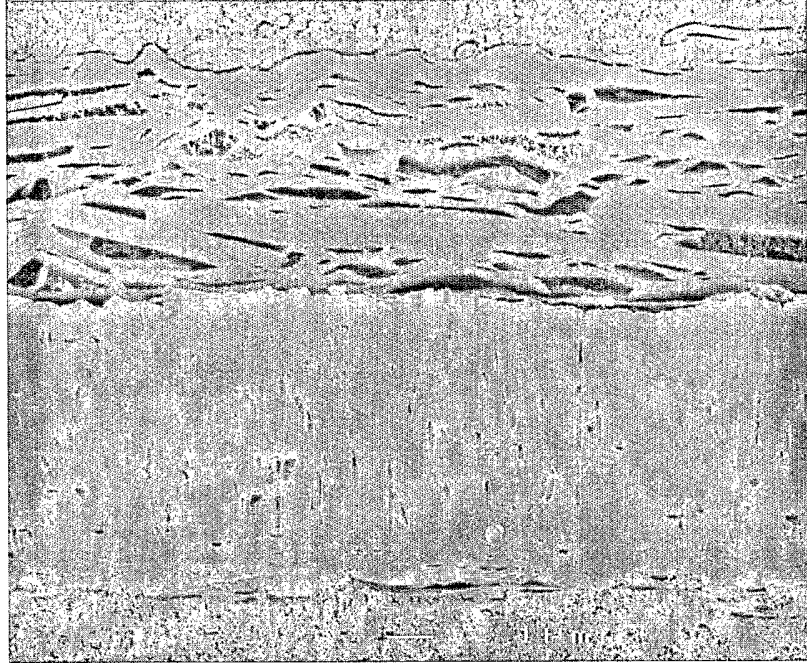
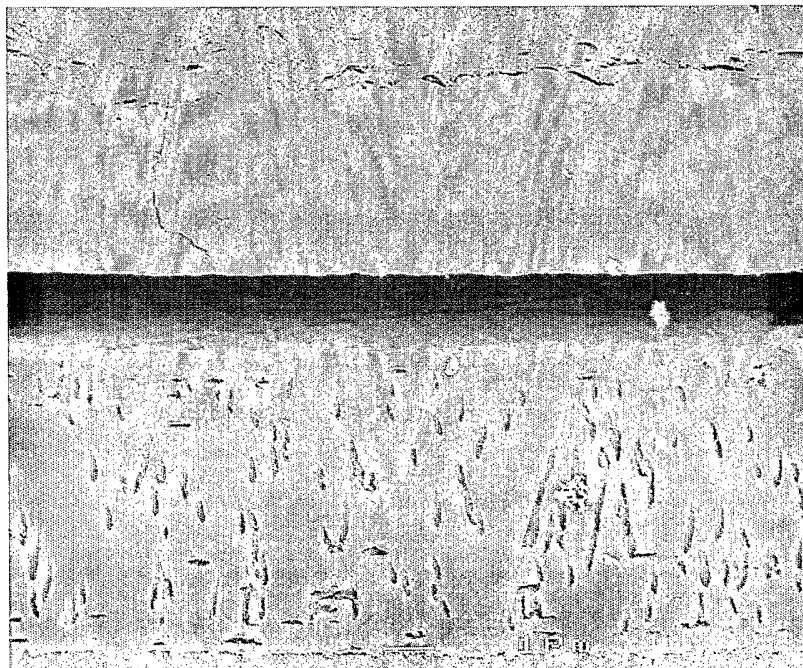


Fig. 3b

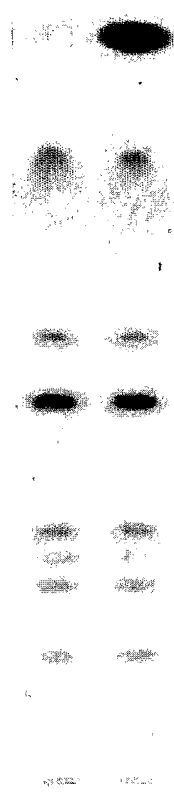


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Fig. 4



Fig. 5



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Fig. 6a

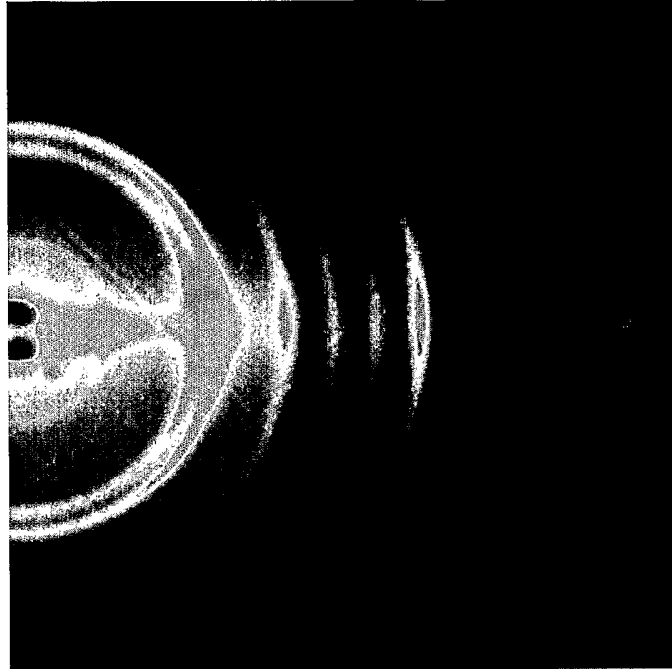
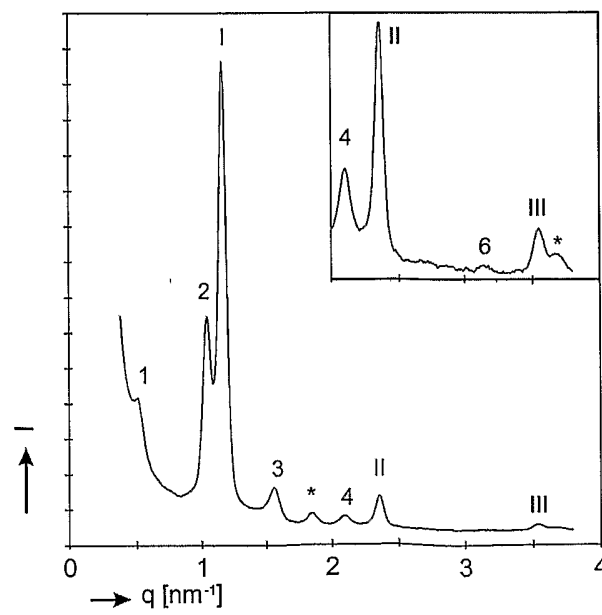


Fig. 6a



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Fig. 7

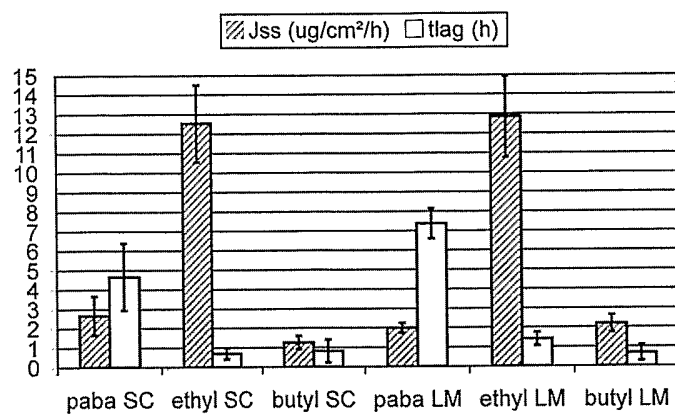
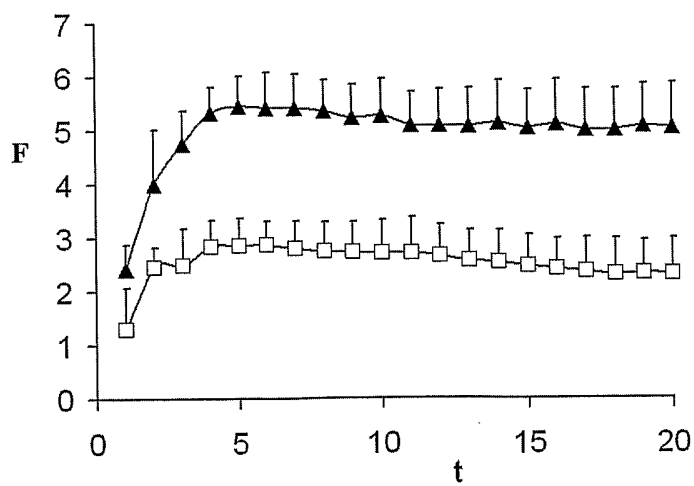


Fig. 8



PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 100-051-71	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/NL2005/000716	International filing date (day/month/year) 05/10/2005	(Earliest) Priority Date (day/month/year) 05/10/2004
Applicant STICHTING VOOR DE TECHNISCHE WETENSCHAPPEN		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
- ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☐ **Unity of invention is lacking** (see Box No. III)

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant
- ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant
- ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. _____

- ☐ as suggested by the applicant
- ☐ as selected by this Authority, because the applicant failed to suggest a figure
- ☐ as selected by this Authority, because this figure better characterizes the invention

b. ☒ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2005/000716

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/92 B01D71/74 B05D1/12

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N B01D B05D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE JAGER M W ET AL: "Modelling the stratum corneum lipid organisation with synthetic lipid mixtures: The importance of synthetic ceramide composition" BIOCHIMICA ET BIOPHYSICA ACTA - BIOMEMBRANES 30 AUG 2004 NETHERLANDS, vol. 1664, no. 2, 30 August 2004 (2004-08-30), pages 132-140, XP002364634 ISSN: 0005-2736 page 134, column 2, paragraph 2.3 ----- -/--	1-15

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* 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
- *L* 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.
- *G* document member of the same patent family

Date of the actual completion of the international search

27 January 2006

Date of mailing of the international search report

01/03/2006

Name and mailing address of the ISA/
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VON EGELKRAUT, R

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2005/000716

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JAECKLE E ET AL: "Comparison of effects of different ointment bases on the penetration of ketoprofen through heat-separated human epidermis and artificial lipid barriers" JOURNAL OF PHARMACEUTICAL SCIENCES 01 JUL 2003 UNITED STATES, vol. 92, no. 7, 1 July 2003 (2003-07-01), pages 1396-1406, XP002364633 ISSN: 0022-3549 page 1397, column 2; table 1	1-15
A	KUEMPEL D ET AL: "In vitro reconstitution of stratum corneum lipid lamellae" BIOCHIMICA ET BIOPHYSICA ACTA - BIOMEMBRANES 1998 NETHERLANDS, vol. 1372, no. 1, 1998, pages 135-140, XP002364635 ISSN: 0005-2736 cited in the application page 136, paragraph 2.1	1-15
A	WO 03/011553 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY; PARAMESWARAN, LALITHA; YOUNG, A) 13 February 2003 (2003-02-13) claims 4-6,12; figure 3	1-15
A	HAUSS T ET AL: "Squalane is in the midplane of the lipid bilayer: Implications for its function as a proton permeability barrier" BIOCHIMICA ET BIOPHYSICA ACTA - BIOENERGETICS 02 DEC 2002 NETHERLANDS, vol. 1556, no. 2-3, 2 December 2002 (2002-12-02), pages 149-154, XP002364636 ISSN: 0005-2728 page 150, paragraph 2.2	4,5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/NL2005/000716

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03011553	A	13-02-2003	NONE