Title: METHOD FOR THE INTRACELLULAR REGENERATION OF HYALURONIC ACID AND COSMETIC COMPOSITION THEREOF

Abstract: Method for the regeneration of hyaluronic acid in cells of connective tissues through administration to a subject in need thereof, of a composition containing liposomes loaded with D-glucuronic acid and N-acetyl-D-glucosamine.
METHOD FOR THE INTRACELLULAR REGENERATION OF HYALURONIC ACID AND COSMETIC COMPOSITION THEREFOR

The present invention relates to a cosmetic method for the regeneration of hyaluronic acid in cells of connective tissues, starting from the hyaluronic acid precursors D-glucuronic acid and N-acetyl-D-glucosamine, or derivatives thereof. More specifically the invention provides a cosmetic composition containing liposomes loaded with D-glucuronic acid and N-acetyl-D-glucosamine, or derivatives thereof, in admixture with a physiologically-acceptable carrier.

Background of the invention

The use of liposomes for the delivery of hyaluronic acid is described in several patent documents. To cite a few examples, WO2006122638 discloses a filler for use in dermocosmetics, containing hyaluronic acid or derivatives thereof structured in phospholipid liposomes. WO03000190 and WO03000191 disclose a method for intra-articular delivery of hyaluronic acid encapsulated in liposomes for the treatment of osteoarthritis. WO9813024 discloses a pharmaceutical formulation containing a mixture of hyaluronic acid and liposomes.

Description of the invention

The present invention is based on the finding of an effective method for regenerating hyaluronic acid inside the cells by administering, to a subject in need thereof, a liposomal preparation containing separately - i.e. not linked by chemical bond - the hyaluronic acid precursors D-glucuronic acid (GA) and N-acetyl-D-glucosamine (GlcNH2), or derivatives thereof. The regeneration of hyaluronic acid inside the cells enables rebuilding the connective tissue damaged by age-related degenerative processes. Such a regenerative effect is particularly useful in dermatology, especially for the treatment of wrinkles and skin-aging.
Accordingly, in a first embodiment the invention provides a cosmetic preparation containing liposomal vesicles loaded with D-glucuronic acid and N-acetyl-D-glucosamine, or derivatives thereof, in admixture with a cosmetically acceptable carrier. As used herein, the term "derivative" indicates an activated form of D-glucuronic acid and N-acetyl-D-glucosamine, preferably the uridindiphospho-derivatives UDP-D-glucuronic acid and UDP-N-acetyl-D-glucosamine. The two active ingredients (GA and GlcNH2 or their derivatives) can be loaded in the same liposomal vesicles or a mixture of vesicles encapsulating either one or the other active ingredient can be used in accordance with the invention. The composition may also contain other ingredients that facilitate the regeneration of hyaluronic acid inside the cells, e.g. by inhibiting its degradation by hyaluronidase. Preferably the hyaluronidase inhibitor is an extract of *echinacea angustifolia*.

The liposomal vesicles may be unilamellar (SUV) or multilamellar (MLV) vesicles of various size (small, medium, large, giant), and may be formed by phospholipids or sphingolipids, preferably by phosphatidylcholine; the vesicles can also contain ingredients modulating their resistance, viscosity or stability.

Methods known in the art can be used to load liposome vesicles with D-glucuronic acid and/or N-acetyl-D-glucosamine or derivatives thereof; for example, the phospholipids may be added to a mixture of the two components, or separately to each single component dissolved in a suitable medium, under constant stirring, followed by turbine homogenization. In cases where the active components are separately added to liposomal vesicles, these are subsequently mixed to form a liposomal mixture of both active ingredients. Once the process is terminated, the liposomes can be dispersed in a cosmetic base or preparation, to form a cream, emulsion, microemulsion, ointment, gel, lotion and oil.
In a preferred embodiment, the liposomal preparation is dispersed in a cosmetic base containing water and glycerol, preferably in the presence of additional ingredients such as EDTA, sodium pyroglutamate, sodium lactate, xanthan gum or similar thickening agents, under conditions suitable for forming a gel paste.

In a further embodiment the invention provides the use of the compositions herein disclosed for dermatological treatments, particularly for the treatment of unaesthetisms such as wrinkles, skin folds, acne scars, post-injury scars, rhinoplasty scars, lips or face (checks and chin) lift.

In a further embodiment the invention provides a method for regenerating hyaluronic acid in human cells and tissues, which comprises applying a liposome preparation as above described, onto said cells or tissues.

Description of Figure 1

PAGEFS electrophoresis of cell cultures; the arrow indicates the disaccharide band corresponding to hyaluronic acid;

S: disaccharide solution indicative of hyaluronic acid;
A-B: control plates;
C: plate treated with placebo liposomes 15 mg/ml;
D: plate treated with placebo liposomes 60 mg/ml;
E-F: plates treated with liposomes loaded with GA and GlcNH2 at 0.5 and 10 mM concentrations, respectively;
G-H: plates treated with liposomes loaded with GA and GlcNH2 at 5 and 50 mM concentrations, respectively.

Example 1 - Preparation of phosphatidylcholine-based liposomal systems for the delivery of D-glucuronic acid (GA) and N-acetyl-D-glucosamine (GlcNH2) and analysis of the content of active ingredients

Materials

Phosphatidylcholine used: PhosPho LCN-DS, soy lecithin, minimum
62% purity, Fancor; Egg phosphatidylcholine (PC), XI-E type, 99% purity, Sigma.

D-glucuronic acid sodium salt monohydrate, N-acetyl-L-glucosamine, Aldrich; Cholesterol, Carlo Erba.

Liposomes were prepared with two methods to increase the encapsulation of AG and GlcNH2.

The following variables were introduced:

- preparation method (direct hydration or hydration after forming a lipidic film)
- quantity and type of phosphatidylcholine
- use of cholesterol
- concentrations of GA and GlcNH2 solutions

**METHOD A (direct hydration):**

The following procedure was followed for 'placebo' and 'loaded' batches:

- weigh the lipophilic phase and add the hydrophilic phase (final volume from 200 µl to 1 ml);
- pass in a vortex until complete phospholipid dispersion and subsequently in termomixer for 15 min at 25°C, 1400 rpm;
- in certain cases maintain the dispersion at 4°C for 24 hrs;
- centrifuge the samples for 60 min at 4°C, 16400 rpm to eliminate the active ingredient that was not encapsulated;
- separate the supernatant from the pellet and maintain the two parts separately freeze at -20°C for subsequent specific dosage of the active ingredients.

By this method, batches formed by phosphatidylcholine PhosPho LCN-DS were produced; the composition of these batches is reported in Table 1. Unless otherwise specified, the hydrophilic phase volume was
maintained at 1 ml. Batches marked with * were hydrated for 24 hrs in a refrigerator prior to centrifugation.

**Table 1**

<table>
<thead>
<tr>
<th>Batch</th>
<th>Phosphatidylcholine amount</th>
<th>Hydrophilic phase</th>
<th>Active ingredient concentration (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JALU 3.7</td>
<td>70 mg</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>JALU 20-22</td>
<td>60 mg</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>JALU 35</td>
<td>600 mg</td>
<td>Water (10 ml)</td>
<td>-</td>
</tr>
<tr>
<td>JALU 42</td>
<td>15 mg</td>
<td>Water (200 μl)</td>
<td>-</td>
</tr>
<tr>
<td>JALU 14-16</td>
<td>15 mg</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>JALU 8-10</td>
<td>7 mg</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>JALU 4-6</td>
<td>70 mg</td>
<td>GA 1.5 mg</td>
<td>6.4</td>
</tr>
<tr>
<td>JALU 23-25</td>
<td>60 mg</td>
<td>GA 0.3 mg</td>
<td>1.3</td>
</tr>
<tr>
<td>JALU 17-19</td>
<td>15 mg</td>
<td>GA 0.3 mg</td>
<td>1.3</td>
</tr>
<tr>
<td>JALU 11-13</td>
<td>7 mg</td>
<td>GA 0.3 mg</td>
<td>1.3</td>
</tr>
<tr>
<td>JALU 36-38</td>
<td>15 mg</td>
<td>GA 1 mg</td>
<td>4.3</td>
</tr>
<tr>
<td>JALU 39-41</td>
<td>15 mg</td>
<td>GA 1 mg (200 μl)</td>
<td>21.5</td>
</tr>
<tr>
<td>JALU 56-58</td>
<td>15 mg</td>
<td>GA 5 mg (200 μl)</td>
<td>105</td>
</tr>
<tr>
<td>JALU 63</td>
<td>75 mg</td>
<td>GA 25 mg</td>
<td>105</td>
</tr>
<tr>
<td>JALU 32-34</td>
<td>60 mg</td>
<td>GlcNH2 0.3 mg</td>
<td>1.35</td>
</tr>
<tr>
<td>JALU 29-31</td>
<td>15 mg</td>
<td>GlcNH2 0.3 mg</td>
<td>1.35</td>
</tr>
<tr>
<td>JALU 26-28</td>
<td>7 mg</td>
<td>GlcNH2 0.3 mg</td>
<td>1.35</td>
</tr>
<tr>
<td>JALU 50-52</td>
<td>15 mg</td>
<td>GlcNH2 5 mg</td>
<td>105</td>
</tr>
<tr>
<td>JALU 59-61</td>
<td>15 mg</td>
<td>GlcNH2 5 mg (200 μl)</td>
<td>113</td>
</tr>
<tr>
<td>JALU 64-66*</td>
<td>15 mg</td>
<td>GA 5 mg (200 μl)</td>
<td>105</td>
</tr>
<tr>
<td>JALU 67-69*</td>
<td>30 mg</td>
<td>GA 5 mg (200 μl)</td>
<td>105</td>
</tr>
<tr>
<td>JALU 71-73*</td>
<td>15 mg</td>
<td>GlcNH2 5 mg (200 μl)</td>
<td>113</td>
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<tr>
<td>JALU 74-76*</td>
<td>30 mg</td>
<td>GlcNH2 5 mg (200 μl)</td>
<td>113</td>
</tr>
<tr>
<td>JALU 77-79*</td>
<td>15 mg</td>
<td>GlcNH2 10 mg (400 μl)</td>
<td>226</td>
</tr>
</tbody>
</table>
METHOD B

Liposome batches were produced using both phosphatidylcholine PhosPhs LCN-DS and PC. Also in this case 'placebo' and 'loaded' batches were produced, according to the following procedure:

- weigh phosphatidylcholine in a round-bottomed flask
- add 500 µl CHCl3 containing 10 mg/ml cholesterol
- dry in rotavapor under reduced pressure at 40°C for 10 min
- rehydrate with 1 ml hydrophilic phase
- vortex for 10 min, centrifuge for 60 min at 4°C, 16400 rpm
- separate sumatant and pellet and freeze at -20°C.

Table 2 reports the liposome batches produced with method B.

<table>
<thead>
<tr>
<th>Batches</th>
<th>Phosphatidylcholine (mg)</th>
<th>Hydrophilic phase</th>
<th>Active ingredient concentration (µmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JALU 43-45</td>
<td>PhosPho LCNDS (15 mg)</td>
<td>GA 1 mg</td>
<td>4.3</td>
</tr>
<tr>
<td>JALU 46-48</td>
<td>PC (10 mg)</td>
<td>GA 1 mg</td>
<td>4.3</td>
</tr>
<tr>
<td>JALU 49</td>
<td>PC (10 mg)</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>JALU 62</td>
<td>PhosPho LCNDS (15 mg)</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>JALU 53-55</td>
<td>Phospho LCNDS 15 mg</td>
<td>GA 5 mg</td>
<td>21</td>
</tr>
</tbody>
</table>

Size determination

A dimensional analysis of the liposome systems was carried out using the laser diffraction apparatus Mastersizer 2000 (Malvern), which operates in a size interval ranging from 20nm to 2mm. The apparatus is equipped with a dispersion unit connected to a pump to shake the sample during the analysis. Each sample was analysed in triplicate and the results are expressed as dio, d50 and d90, i.e. the diameter of 10%, 50% and 90% of the analysed sample.

Table 3 reports the analysis carried out on placebo batches at different concentrations of phosphatidylcholine, prepared according to each method.
The results evidence the formation of vesicles of micrometric size; as far as PhosPho LCNDS is concerned, the size is constant using either of the two methods with the same amount of phospholipids; the results of sample Jalu 49 indicate that the size depends on the purity of the starting phospholipid.

Example 2 - gel preparation

Phase A (Liposome formation)

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000 rounds/min) and with a paddle agitator, in the order:

- DEMINERALIZED WATER 56.400%
- GA 0.100%
- GlcNH2 1.100%

the components are mixed until a complete dissolution is obtained.

Phase A' is added slowly under turbine (2.500 rounds/min):
- LECITHIN 1.500%

homogenization is carried out for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

Phase B

In a separate steel reactor equipped with heating, stirring and turbine,
the following solution is prepared:

- DEMINERALIZED WATER 30.000%
- DISODIUM EDTA 0.500%
- GLYCEROL 8.000%
- SODIUM PYROGLUTAMATE 1.000%
- SODIUM LACTATE 1.000%

the solution is shaken until it becomes homogeneous and the mass is heated at 70°C, then Phase B' is added slowly under the turbine (1.500 rounds/min):

- XANTHAN GUM 0.400%

homogenization is carried out until complete dispersion of xanthan gum with formation of a compact and homogeneous gel. The mixture is cooled to 25°C and the Phase A is poured under stirring. Stirring is continued for additional 20 min until a homogeneous and compact mass is obtained.

Example 3 - gel preparation (alternative 1)

**Phase A (1st liposome)**

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000 rounds/min) and with a paddle agitator, in the order:

- DEMINERALIZED WATER 18.800%
- GLUCURONIC ACID 0.100%

the components are mixed up to complete dissolution. LECITHIN (0.500%) is added slowly under turbine (2.500 rounds/min). Homogenization is continued for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

**Phase A' (2nd liposome)**

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000
rounds/min) and with a paddle agitator, in the order:

- DEMINERALIZED WATER 18.800%
- N-ACETYL-L-GLUCOSAMINE 1.100%

the components are mixed up to complete dissolution. LECITHIN (0.500%) is added slowly under turbine (2.500 rounds/min). Homogenization is continued for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

**Phase A" (3rd liposome)**

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000 rounds/min) and with a paddle agitator, in the order:

- DEMINERALIZED WATER 18.800%
- Echinacea angustifolia (dry extract) 0.200%

the components are mixed up to complete dissolution. LECITHIN (0.500%) is added slowly under turbine (2.500 rounds/min). Homogenization is continued for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

**Liposome preparation**

In an inox-steel reactor equipped with agitator, the three phases A, A' and A" are mixed slowly for 10 min until complete homogenization is obtained.

**Phase B**

In an inox-steel reactor equipped with heating, stirring and turbine the following solution is prepared:

- DEMINERALIZED WATER 29.800%
- DISODIUM EDTA 0.500%
- GLYCEROL 8.000%
- SODIUM PYROGLUTAMATE 1.000 %
- SODIUM LACTATE 1.000%

The mixture is stirred up to homogeneity and heated at 70°C. Once the temperature is reached, Phase B is added slowly under turbine (1.500 rounds/min):

- XANTHAN GUM 0.400%

Homogenization is continued until the dispersion of Xanthan gum is complete and a homogeneous and compact gel is obtained. The temperature is cooled to 25°C and the mass is added to the liposome preparation under stirring. Stirring is continued for 20 min until a homogeneous and compact mass is obtained.

Example 4 - gel preparation (alternative 2)

Phase A (1st liposome)

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000 rounds/min) and with a paddle agitator, in the order:

- DEMINERALIZED WATER 18.800%
- UDP - D-GLUCURONIC ACID 0.100%

the components are mixed up to complete dissolution. LECITHIN (0.500%) is added slowly under turbine (2.500 rounds/min). Homogenization is carried out for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

Phase A' (2nd liposome)

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000 rounds/min) and with a paddle agitator, in the order:

- DEMINERALIZED WATER 18.800%
- UDP-N-ACETYL-L-GLUCOSAMINE 1.100%

the components are mixed up to complete dissolution. LECITHIN
(0.500%) is added slowly under turbine (2.500 rounds/min). Homogenization is continued for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

**Phase A" (3rd liposome)**

The following components are exactly weighed and placed in a inox-steel reactor equipped with a variable-speed turbine (0-3000 rounds/min) and with a paddle agitator, in the order:

- **DEMINERALIZED WATER** 18.800%
- **Echinacea angustifolia** (dry extract) 0.200%

The components are mixed up to complete dissolution. LECITHIN (0.500%) is added slowly under turbine (2.500 rounds/min). Homogenization is continued for 15 min. The turbine is stopped and mixing is continued with the paddle agitator for additional 10 min.

**Liposome preparation**

In an inox-steel reactor equipped with agitator, the three phases A, A' and A" are mixed slowly for 10 min until complete homogeneity is obtained.

**Phase B**

In an inox-steel reactor equipped with heating, stirring and turbine the following solution is prepared:

- **DEMINERALIZED WATER** 29.800%
- **DISODIUM EDTA** 0.500%
- **GLYCEROL** 8.000%
- **SODIUM PYROGLUTAMATE** 1.000%
- **SODIUM LACTATE** 1.000%

The mixture is stirred up to homogeneity and heated at 70°C. Once the temperature is reached, Phase B' is added slowly under turbine (1.500 rounds/min):

- **XANTHAN GUM** 0.400%
Homogenization is continued until the dispersion of Xanthan gum is complete and a homogeneous and compact gel is obtained. The temperature is cooled to 25°C and the mass is added to the liposome preparation under stirring. Stirring is continued for 20 min until a homogeneous and compact mass is obtained.

Example 5 - set up of a cellular system for the in vitro evaluation of the liposome systems

The studies on the cellular effects were carried out with NHDF cells (Normal Human Dermal Fibroblasts), 6 passages in DMEM, 10% FBS, 1% PenStrep, 1% glutamine, in multiwell plates (growth area for single well = 9.6 cm²). Each well contained 300,000 confluent cells.

Samples were inoculated according to the following scheme, in sterile conditions and under laminar-flow at 37°C placing 2 ml medium in each well and the wells were incubated at 37°C for 60 hrs, 5% CO2, 97% humidity.

Scheme of the samples inoculated in 8 plates:

> A-B control plate;
> C plate treated with liposomes (control) 15 mg/ml;
> D plate treated with liposomes (control) 60 mg/ml;
> E-F plates treated with liposomes loaded with GA and GlcNH2 at 0.5 and 10 mM concentrations, respectively.
> G-H plates treated with liposomes loaded with GA and GlcNH2 at 5 and 50 mM concentrations, respectively.

At the end of the scheduled time period, plates were analysed with phase-contrast optical microscopy, whereby cell death was found in plates D, E, F, G, H. Sumatants were recovered, centrifuged and examined for their hyaluronic acid content. In brief, 1 ml clarified medium was lyophilized, taken up with 300 ml ammonium acetate pH 7.0 and precipitated with 4 volumes of cold ethanol and left at -20°C for 16-18 hrs, centrifuged and the pellet was
recovered; this step was repeated a second time. At the end of the second precipitation, the pellet was completely dried and digested with 100 mil/ml hyaluronidase SD at 37°C for 1 h and with 100 mU/ml Condroitinase ABC at 37°C for 3 h. The samples were then liophilized and derivatized with 2-aminoacridone (AMAC).

40 µl aliquots of 12.5 mM AMAC solution in acetic acid/DMSO (3:1 7, v/v) were added to each sample and incubated for 10-15 min at room temperature; then 40 µl of 1.25 M NaBH3CN fresh solution were added and the samples incubated for 16-18 hrs at 37°C. After the derivatization, the samples were separated by PAGEFS.

Figure 1 shows that the disaccharide band, which is indicative of hyaluronic acid synthesis, is present in samples containing liposomes and results particularly thicker, which means that a higher concentration of hyaluronic acid is formed, in the samples containing a higher concentration of GA and GlcNH2 precursors.

In Figure 2 the results are expressed as % hyaluronic acid production with respect to the control, assumed 100%.

Conclusions

The incubation time in this experiment (60 hrs) resulted too long for cell survival.

The electrophoresis shows that the samples treated with phospholipid concentrations of 15 and 60 mg/ml do not present inhibitory effect on the biosynthesis of hyaluronic acid.

The inoculum of liposomes containing GA and GlcNH2 at 5 and 50 mM concentrations, respectively, induce an increase of the disaccharide band which is indicative of hyaluronic acid synthesis.

Example 6 - In vitro efficacy study

Based on the previous results, the following method was carried out:
NHDF (Normal Human Dermal Fibroblasts) cells, 6 passages in DMEM, 10% FBS, 1% PenStrept, 1% glutamine, in multiwell 6 plates (growth area for each single well = 9.6 cm²). Each well was loaded with 300,000 confluent cells.

Samples were inoculated in sterile conditions, at 37°C with 2 ml medium added to each well and incubated at 37° for 48 or 72 hours, 5% CO₂, 97% humidity. 12 hrs after the inoculum, fresh medium was added.

Samples inoculated in 12 plates:
- 2 control plates 48hr;
- B-C plates treated for 48 hrs with liposomes loaded with GA and GlcNH₂ at 0.25 and 5 mM concentrations, respectively;
- H-I plates treated for 48 hrs with liposomes loaded with GA and GlcNH₂ at 0.5 and 10 mM concentrations, respectively;
- 2 control plates 72 hrs;
- D-E plates treated for 48 hrs with liposomes loaded with GA and GlcNH₂ at 0.25 and 5 mM concentrations, respectively;
- F-G plates treated for 48 hrs with liposomes loaded with GA and GlcNH₂ at 0.5 and 10 mM concentrations, respectively.

At the end of the scheduled time period, plates were analysed with phase-contrast optical microscopy and living cells were counted.

The analysis of hyaluronic acid produced by cells is carried out on the medium treated with specific enzymes (hyaluronidase and condroitinase) by means of a chromatographic analysis specific for proteins (FPLC). The separation is carried out on a pre-packed column Superdex 200HR 10/30 at room temperature, with a flow rate of 0.35 ml/min; ammonium acetate 0.1 M pH7, containing 0.05% Tween 20 was used as the buffer. The UV-Vis detector was set at 280 nm.

The results for cell viability, expressed as percentage of vital cells
compared to controls, are reported in Figure 3. Particularly positive results were obtained with plates B-C and D-E wherein viability resulted higher than 80%.

The results in terms of production of hyaluronic acid at 48 and 72 hrs are reported in Figure 4. The results are expressed as HA% determined as the ratio of the amount of HA found in cells treated with liposomes to the amount produced by control cells.

The results demonstrate that the production of hyaluronic acid is by far higher in treated plates than in controls; this indicates that the liposomal systems effectively deliver the two hyaluronic acid precursors into fibroblasts, leading to the (re)constitution of this essential component of derma structure and skin.

Example 7 - in vivo efficacy study using non-invasive methods for cutaneous bioengineering

Population: 20 healthy volunteers (males and females) were selected on the basis of the following general exclusion criteria:

- individuals presenting, in the investigated cutaneous area, signs that could interfere with the assay results; hypersensitivity or intolerance known for cosmetic products and/or products for body cleaning/detersion, including sunscreens; sun-exposure susceptibility; subjects treated with photosensitive drugs less than one month earlier; individuals treated with UVA and/or UVB radiations less than one month earlier.

**Tested formulations**

Formulation A: glucuronic acid 0.1%, acetyl glucosamine 1.1%, phosphoLCN-DS 1.5%, EDTA 2Na 0.5%, glycerol 8%, PCA Na 1%, Na lactate 1%, Sepiplus 400 2.3%, demineralized water q.s. to 100.

Formulation B, used as placebo: phospho LCN-DS 1.5%, EDTA 2Na 0.5%, glycerol 8%, PCA Na 1%, Na lactate 1%, Sepiplus 400 2.3%,...
demineralized water q.s. to 100.

Anatomic area of application
The subjects, divided in groups A and B, were requested to apply the formulation A or B, respectively, to the left forearm, whereas right forearms were used as controls.

Dose and administration
The formulations were applied once a day

Treatment period. 2 months

Biometric evaluation
The following parameters were considered in this study: water content of horny layer, cutaneous firmness (RO), total (R2) and neat elasticity (R5), erythema degree, skin-feeling. The measurement of cutaneous parameters was carried out at the beginning of the experiment (t0) and after 15 (t1), 30 (t2) and 60 (t3) days by means of the multi-probe Cutometer® MPA 580 (CK electronic GmbH, G.F. Secchi, CO), according to the international guidelines.

Simultaneous application of different products
The subjects were advised not to apply other cosmetic products to the treated areas, 3 days in advance and during the period of study, to avoid uncertain results.

Results
Erythema index
In order to ascertain the tolerability of the applied formulations on skin, the erythema level on the treated and control sites was evaluated, for all the time periods checked during the study. It is known that an increase of the erythema level following the application of a formulation indicates scarce tolerability of the formulation itself.

The results obtained in this study are herein reported as erythema indexes, i.e. as ratio between the erythema level at time t and the basic
erythema level for every site examined.

When comparing the histogram referred to the application site with the control (Figure 5) for each time period, no significant differences may be noted; therefore, no increase of the erythema level may be associated to the application of the two formulations.

Further, when observing the graphs obtained for each formulation in time, it is noted that the erythema indexes keep close to unit for all times taken into consideration: the application of the two formulations does not cause a change of the erythema level even after a prolonged application.

Therefore, both the formulations used in this study are well tolerated and no irritation can be ascribed to the vehicle, to liposomes or to the active ingredients at the studied concentrations.

Skin hydration

The table 4 below reports the average hydration values of the population under exam obtained with a corneometer as well as the change with respect to the forearm used as control at each time.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Todays</th>
<th>T 30days</th>
<th>T 60days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formulation A</strong></td>
<td>average ± s.d.</td>
<td>(+34.05)</td>
<td>(+38.88)</td>
</tr>
<tr>
<td></td>
<td>% vs. control</td>
<td>(+38.18)</td>
<td>(+28.96)</td>
</tr>
<tr>
<td><strong>Formulation B</strong></td>
<td>average ± s.d.</td>
<td>(+35.88)</td>
<td>(+22.88)</td>
</tr>
<tr>
<td></td>
<td>% vs. control</td>
<td>(+24.08)</td>
<td></td>
</tr>
</tbody>
</table>

By comparing the corneometry values from forearms treated with the corresponding controls, it is noted that the significant increase appears very similar after 15 days application (34.05% and 35.88%). From the above data, it is evident that the application of both formulations is accompanied by an
increase of water content in the corneal layer, wherein such hydration improvement can be ascribed to the composition of the formulations and to the presence of liposomes, regardless of the active ingredients contained therein.

Following repeated applications of formulation A for 30 days, an increase is obtained 66.87% higher than the increase due to the application of formulation B. The greater increase of surface hydration after repeated application of formulation A is probably due to the presence of active ingredients inside liposomes.

Such hydration increase in subjects belonging to group A is not maintained as such after 60 days daily application (28.96% vs. 24.08% of group B). This result may be due to a stability problem of the active ingredients contained in the liposomal structures.

The study results are reported in Figure 6 as hydration indexes, i.e. as ratio between the values obtained at different times \( t \) and the basal values \( (TO) \).

Even by comparing the results with the basal values, skin hydration increase following application of the formulations may be observed at all times evaluated in this study.

**Skin elasticity**

Skin elasticity and firmness change after application of the formulations is assayed by means of a software programmed to carry out a number of operations with curves obtained after measuring the analyzed area. Exemplary curves obtained during the study are reported in Figure 7.

The following, internationally-approved parameters for the evaluation of elasticity and firmness, may be obtained from such curves:

\[ R_0 = \text{highest point of the first curve; skin firmness index;} \]

\[ R_2 = \text{curve portion comprised between the maximum width and the} \]

skin ability to re-deform. This parameter is very important since it indicates the total skin elasticity.

\[ R_5 = \text{This parameter indicates the actual elasticity, that is the total elasticity minus the skin viscoelastic portion.} \]

The results relating to this three parameters will be discussed separately.

As to skin firmness, Figure 8 reports the variation of the RO index with respect to the starting value, for both tested formulations, in treated and control areas.

The results show that after 15 days treatment, both formulations have a definitively higher index with respect to the non-treated areas, showing a clear improvement in skin firmness; after 30 or more days treatment, the improvement is evident only for formulation A, which contains the active ingredients.

These results are perfectly consistent with the results relating to skin hydration.

As to skin elasticity parameters, Figure 9 shows the percent variation of indexes R2 and R5 compared to the non-treated area, for both the tested formulations.

The results are in line with those reported above: after 15 days treatment, a positive effect due to the application of formulations containing liposomes may be observed whereas, in case the application is prolonged, the specific action of glucuronic acid and of glucosamine contained in liposome systems may result in a consistent increase of skin elasticity.

**CONCLUSIONS**

1. liposomes containing glucuronic acid and acetyl-glucosamine have suitable characteristics of size and active-ingredients content.

2. the in vitro results clearly show an increase in the amount of
hyaluronic acid produced by cells after incubation of the precursor-containing liposomes, thus pointing out the ability of liposomal systems to deliver active ingredients inside fibroblasts.

3. the formulation containing glucuronic acid and acetyl-glucosamine proved well-tolerated after a continued two-month use; such formulation proved also capable to remarkably improve skin hydration, firmness and elasticity parameters after 30 days treatment with respect to the formulation containing placebo liposomes.
CLAIMS

1. A cosmetic preparation containing liposome vesicles loaded with D-glucuronic acid and N-acetyl-D-glucosamine, or a derivative thereof selected from UDP-D-glucuronic acid and UDP-N-acetyl-D-glucosamine, in admixture with a cosmetically-acceptable carrier.

2. A preparation according to claim 1, further containing a hyaluronidase inhibitor.

3. A preparation according to claim 2, wherein said hyaluronidase inhibitor is an extract of *echinacea angustifolia*.

4. A preparation according to claims 1-3, wherein the vesicles consist essentially of phosphatidylcholine.

5. A preparation according to claims 1-4, which is in the form of cream, emulsion, microemulsion, ointment, gel, lotion or oil.

6. A preparation according to claim 5, which is in the form of a gel containing water and glycerol.

7. A preparation according to claim 6, further containing EDTA, sodium pyroglutamate, sodium lactate and a thickening agent, preferably xanthan gum.

8. The use of liposomes encapsulating D-glucuronic acid and N-acetyl-D-glucosamine, or derivatives thereof, for the preparation of a cosmetic composition.

9. The use of a preparation according to claims 1-7, for the cosmetic treatment of wrinkles, skin folds, acne scars, post-injury scars, rhinoplasty scars, lips or face (checks and chin) lift.

10. A method for regenerating hyaluronic acid in human cells or tissues, which comprises applying a preparation according to claims 1-8 onto said cells or tissues.
Figure 1
Figure 2

![Bar chart showing % HA for control and various samples (C, D, E, F, G, H).]
Figure 4

![Bar chart showing the produced HA vs control (in %) for different categories: B-C, H-I, D-E, and F-G. The chart illustrates varying levels of production across these categories.]
Figure 5

[Graph showing Erythema index with sites A treaty, A control, B treaty, and B control compared across three different groups: 15 gg, 30 gg, and 60 gg.]
Figure 6
Figure 8

The graph shows the comparison of RO t/10 index for different treatments and controls. The treatments and controls are labeled as follows:

- A treaty
- A control
- B treaty
- B control

The graph includes data points for 15 gg, 30 gg, and 60 gg conditions.
A. CLASSIFICATION OF SUBJECT MATTER

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

B. RELDS SEARCHED

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

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

EPO-Internal, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category| Citation of document with indication, where appropriate, of the relevant passages| Relevant to claim No
---|---|---
Y| EP 0 295 092 A (UNILEVER PLC [GB]); UNILEVER NV [NL]) 14 December 1988 (1988-12-14) page 12, line 57 - line 63; claim 1; examples 1,4-7 | 1,5,6,9
Y| WO 95/24497 A (FIDIA ADVANCED BIOPOLYMERS SRL [IT]; LANSING MANFRED [DE]; FREHM PETER) 14 September 1995 (1995-09-14) claims 1,16 | 1,5,6,9

Date of the actual completion of the international search: 13 March 2009

Date of mailing of the international search report: 23/03/2009
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   Although claim 10 is directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.
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