CHITIN-NANOFIBRILS: A NEW ACTIVE COSMETIC CARRIER

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Summary

The cosmetic industry has evolved over the last 50 to 60 years from an era of secret formulas, elusive promises and false hope to an entirely new industry based on science. No longer are the cosmetic giants isolated scions, but there is an ever growing interactions and interdependence among cosmeceutical, pharmaceutical, biochemical, and medical community. Thus new development has been successfully translated into more effective treatments as well as preventive treatments of dry or aging skin, as example.

At this purpose, active compounds of new cosmetic products has to be carefully selected to obtain the best efficacy and safeness. Thus innovative cosmetics have become more sophisticated in both formulations and presentation meanwhile new tests have been developed to ensure not only quantity and safety but also the validation of products claims.

Different in vitro and in vivo tests are reported to show the capacity of chitin nanofibrils to be used both as penetration enhancer and active compound as anti-ageing agent.

Riassunto

Negli ultimi 50 anni l'industria cosmetica si è trasformata da produttrice di formule basate su false promesse a produttrice di prodotti innovativi basati su studi scientifici. La cosmetologia è così diventata scienza multidisciplinare collegata con la biologia, la farmaceutica e con la comunità medica.

Sono state prodotte con successo nuove formulazioni utili per impedire, ad esempio, l'instaurarsi di xerosis o per prevenire il fotoinvecchiamento cutaneo.

A questo scopo è indispensabile che siano attentamente selezionati sia i principi attivi che i veicoli
utilizzati per ottenere i migliori risultati di efficacia e sicurezza d’uso dei prodotti cosmetici formulati. Sono nati così cosmetici innovativi più sofisticati sia nelle formulazioni che nelle loro prestazioni, mentre contemporaneamente sono state sviluppate metodologie di controllo necessarie per supportarne i relativi messaggi pubblicitari.

In questo lavoro vengono riportati studi in vitro ed in vivo necessarie ad evidenziare le capacità possedute dalle nanofibrille di chitina quali promotrici della penetrazione transcutanea e quale principio attivo utile, ad esempio, per la formulazione di cosmetici antietà.
INTRODUCTION

The central tenets of current and upcoming molecular biology, nanoscience and innovative ingredients will continue to play an ever increasing role in the cosmetic and skin care industry (1-3). On one hand the increasing demand for anti-aging personal care products and the need to understand these products' mechanism of action continuously provides a scientific challenge to the industry formulator and expert evaluator (4,5).

On the other hand informed consumers want to know which topical treatments are a viable alternative to invasive surgical procedures, and which active ingredients and carrier systems are able to preserve and even regenerate a youthful, healthy look. Numerous studies show, in fact, that present day personal care products provide the greatest efficacy only after months of regular and repeated application (6). Thus the technology advanced delivery and controlled-release of highly functional ingredients, offered by the new chitin-nanofibril, makes them the most reliable way to achieve those desired results (7-9).

Aims

We aimed to examine both in vitro and in vivo the activity of some well known antioxidant compounds transported through the skin layers by a nano-emulsion carrier based on chitin-nanofibrils, previously used in our group's research (10-12).

In order to understand the mechanism of action of these compounds against free radicals and pollutants at the skin level, we compared a mixture of melatonin, lutein and ectoin pre-linked with chitin-nanofibrils (CN) and embedded in a CN-nano-emulsion, in accord with other studies already published by our group (13,14).

MATERIALS AND METHODS

Experimental Section

METHODS

In Vitro Activity.

Regenerative activity

As it is known, the dermis represents the fundamental and supporting portion of the skin. Its papillary portion contains a high amount of collagen and elastic fibers required to give firmness and elasticity to the skin. Fibroblasts, contained in high amounts in the papillary dermis, continuously produce these fibers (15). Cutaneous aging causes a thinning of the dermis and a qualitative and quantitative reduction of the fibroblasts, which no longer produce collagen efficiently. Thus the effect of chitin nanofibrils on the growth of a fibroblast culture was tested both alone and in association with some antioxidant and immunostimulant compounds.

The skin regeneration process is, in fact, very efficient in young people and in healthy skin, but drastically reduced with aging. It is also influenced by stress, loss of sleep, and air conditioning, capable of reducing the normal cellular turnover, and increasing both age spots and wrinkles (16). All these phenomena, contributing to the general aging of skin, may be examined with different methods, e.g. by assessing the energy required for various metabolic processes, stored as ATP; or, by measuring fibroblast activity and collagen and melanin synthesis, thereby verifying the hyperpigmentation activity that contribute to the formation of age-spots.

Fibroblast activity

Fibroblasts of NB1RGB strain were used (2 x 105 cell/ml) and suspended in the α-MEM culture medium placed in 8 Petri dishes (containing
10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin) (17). To 6 cultures were added, respectively a 10 mg/ml concentration of:
1. melatonin;
2. chitin-nanofibrils (CN)
3. melatonin-lutein;
4. melatonin-ectoin;
5. melatonin-lutein-ectoin;
6. melatonin-lutein-ectoin-chitin-nanofibrils while two were left as control.
The results obtained are reported in Fig. 1, illustrating the medium percent of cell proliferation with respect to the control value.
All biochemical processes require energy that is accumulated in the form of ATP (adenosyn-triphosphate). ATP was measured on a culture of keratinocytes irradiated with 4J/cm² UVA + 04 J/cm² UVB (SOL500 lamp – Munich, Germany) and compared with ATP levels on keratinocytes irradiated and additioned with the products under study. As known, irradiation causes a drastic reduction of the ATP present and it is dose-dependent (18).

**Measuring ATP activity**

Of the 8 dish cultures, 6 received 10 µg/ml of the different substances to be tested 24 hours before UV irradiation, whereas two served as control. The ATP level was detected by using ATP Lite-M (Chemiluminescent kit, Packard).
Results are reported in Fig. 2, illustrating the residual medium percent amount of ATP per dish.
**Stimulation of collagen synthesis**

A continuous and regular collagen synthesis is of fundamental importance for the ECM (extracellular matrix) structure, and therefore for skin elasticity, firmness and wrinkle reduction. A decrease in the rate of collagen Type-I production, and expression of the genes coding for collagen Type-II and III, can be observed during aging. The rate of collagen Type-I secretion was measured by the use of specific antibodies (Elisa method) on 8 cell cultures, 6 of which enriched with 10 µg/ml of the various substances directly introduced in the culture medium. Two served as control. Measurements were done after 6 days of incubation. Results are reported in Fig.3, illustrating the medium percent increase of collagen with respect to the control value.

**Depigmentation activity**

The depigmentation activity present in the various mixtures of products was verified on B16 melanoma cells (5x10 cell/ml) suspended in MEM culture medium (10% FBS, 1000 I.U. (International Units) /ml penicillin and 100 µg/mg streptomycin) containing 2 mM theophylline. The suspension was subdivided into 8/500 μl portions. To each portion, placed in suitable bars, 50 µg/ml of the various mixtures of active agents were added. Two were the untreated controls. Post-incubation: 300 µl PBS were added, then all samples were ultrasonicated. An increase or decrease in the presence of melanin was measured by 415-nm spectrophotometer (20). The various compost examined showed significant decreases in melanin formation.
The average results obtained are reported in Fig.4, illustrating percent values with respect to the control value.

**In Vivo Activity. Skin absorption -potential**

Before starting the other *in vivo* studies, the absorption potential of CN was controlled, as probable skin penetration enhancer of our active compounds, in comparison with the vehicle alone. The dansyl chloride labelling technique was used, in keeping with our previous experiences (21-24). A 5% dansyl chloride concentration finely triturated was added into four formulations:

1. vehicle alone
2. vehicle + CN
3. melatonin-lutein-ectoin + vehicle(product B)
4. melatonin-lutein-ectoin-CN + vehicle (product A).

The formulations were applied indifferently on the right (product 1 and 2) and/or the left (product 3 and 4) volar forearm of 10 women volunteers, and kept under semi-occlusive dressings for 24 hours. The day after, the area was cleansed using a lotion (Idroskin latex) and soft tissue paper. The dried stratum corneum (SC) surface was removed using 15 strips of an adhesive tape (Sellotape®) in succession.

Using the correct methods it is possible to obtain successive layers of the stratum corneum, each one of single thickness. On all the SC-layers, the level of fluorescence was measured by UV illumination, using an arbitrary scale of 0-8. The level of redox-balance (not reported) was also detected on the different layers treated by mixture 3 and 4, in accord with our previous studies (23,24).
The obtained mean final results are reported in Fig. 5.
External aggressive agents provoke an immune response, always accompanied by inflammatory reactions. When these reactions are excessive, the cascade that produces the pro-inflammatory mediators can become abnormal. Interleukin 8 (IL-8) is the one most responsible for the permanence of the inflammatory state (25).

**Anti-Inflammatory Effect**

**IL-8 examined**

10 volunteers suffering from cutaneous dryness of atopic origin, ranging in age from 15 to 20, with elevated interleukin 8 (IL-8) expression were selected. Then lymphocytes were isolated. The 8 collected blood samples of lymphocytes, 6 pre-supplemented and 2 non-supplemented (control) with the components under study (1 µg/ml), were subdivided into 8 Petri dishes, and further supplemented with 10 mg/ml Tumor Necrose Factor α (TNF-α). The TNF-α addition caused a marked increase in IL-8 production, whereas the substances under study were supposed to reduce said increase. IL-8 amounts were determined photometrically, by specific antibodies (26,27).

The results obtained are reported in Fig. 6, illustrating the relative medium amounts of free IL-8 with respect to the control values.

**Experimental Project**

To a group of 40 women volunteers ranging in age from 25 to 35, suffering from photoaged dry skin, were distributed under double-blind conditions two different typologies of cream to be applied by light massage indifferently on the left or right arm, in the morning and in the evening, as well as on the two hemi-sides of the face.
The two creams, contained in differently-coloured tubes, were sufficient for 60 days of treatment.

Initially and throughout the entire experiment the groups were subdivided as follows:

10 women treated with product (emulsion carrier with melatonin –lutein-ectoin-CN) and carrier only, (product 1)
10 women treated with product (emulsion carrier with melatonin –lutein-ectoin) and carrier only, (product 2)
10 women treated with emulsion with CN and carrier only (product 3)
10 women treated with the carrier only (group 4) (control group)

15 days before, at the starting and 60 days after the control period, skin surface lipids, hydration, and TEWL, were verified by the 3C System (28); the lipid peroxides by the MDA method (29) All the valves were the average of 3 assessments.

**MEASUREMENT EQUIPMENT**

**Skin surface lipids**

The skin surface lipid levels were measured with the 3C System (Dermotech S.r.l., Rome, Italy). Determination is based on photometric measurement of light transmission through a skin surface imprint obtained by applying a frosted pastic foil to the designated skin area. It allows adherence of skin lipids in a 1 cm² area calculated digitally in µg/cm² (28).

**Skin hydration**

The hydration of the horny layer was assessed by measuring electrical capacitance of the skin surface by means of the 3C System.
When the probe was applied to the skin (recording time 0.5 sec.), the capacitance was displayed digitally in arbitrary 3C units. The results reported are expressed as mean values of the measurements performed on four different right or left sites (check, forehead, chin and nose).

**Transepidermal water loss (TEWL)**

All evaluations were performed after a 30-minute acclimatization period in a room at 22±2°C with 50% humidity. Water evaporating from the skin surface was measured quantitatively with the 3C System methodology. The 3C System probe consists of a cylindrical open chamber measuring system, with diameter 14 mm, height 10 mm and a distance from skin area 0.95 cm². Two sensor units, containing thin capacitative film transducers, were placed in the probe at 3 and 7 mm distance from the skin surface. TEWL is calculated digitally in g/m² h.

**Lipid peroxides**

Lipid peroxides were checked determining the presence of these derivatives by the malonyl dialdehyde (MDA) method (29). The global results obtained are reported in Fig.7-10, illustrating the percent decrease of skin lipid peroxides and TEWL (Fig. 7 and 8) and the percent increase of skin hydration, and superficial skin lipids (Fig 9 and 10). All the values were calculated with respect to the starting values.

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**IL-8 checking on lymphocytes from volunteers' blood pre-treated with Chitin nanofibrils in mixture with antioxidant and immunomodulant compounds**

![Graph showing IL-8 levels](Image)

Fig. 6

All p values are highly significant as control (p<0.005) and significant as to groups (p<0.05).
Figure 8: After 60 days of bi-daily treatment on facial skin TEWL recovers on women affected by dry skin activity of chitin nanofibers, antioxidant and immunomodulatory compounds on

Figure 7: Lipid peroxides found in volar forearm of women affected by dry skin activity of chitin nanofibers, antioxidant and immunomodulatory compounds on
After 60 days of daily treatment on facial skin
on surface skin, lips, and women aged by dry skin.
Activity of Chitin nanofibrils, antioxidant and immunomodulators compounds
**Anti-irritation effect**

A sub-clinical skin erythema, obtained from a 1MED of UVB irradiation (by a Multiport 601 150W Solar Light Simulator, Solar Light Co, INS Philadelphia, PA, USA), was measured by a laser Doppler. It is considered the most sensitive method for measuring sub-clinical changes in the skin’s microcirculation (30, 31). Depending on the site being analyzed, subjects were required to be seated, or to recline, during the equilibrium period.

During testing, the probe applied to the skin surface using double sided adhesive tape, was maintained in position for at least 30 second and measurements were repeated at least two times, averaging the data.

In keeping with the test procedure the skin of the volar forearm was divided in 8 areas of 1 cm² each.

- a) untreated
- b) placebo treated
- c) pre-treated by product A (emulsion carrier of melatonin-lutein-ectoin-CN) prior to UV-exposure (preventive treatment)
- d) pre-treated by product B (emulsion carrier of melatonin-lutein-ectoin) prior to UV-exposure (preventive treatment)
- e) post-treated by product A soon after UV-exposure (curative treatment)
- f) post-treated by product B soon after UV-exposure (curative treatment)
- g) post-treated 2 hours after UV-exposure by the product A (delayed curative treatment)
- h) post-treated 2 hours after UV-exposure by the product B (delayed curative treatment)

An increase in microcirculation is correlated to an increase in sub-clinical erythema, whereas a decrease in microcirculation is indicative of a decrease in clinical erythema. The obtained results are reported on the Fig.11.
STATISTICAL ANALYSIS

The Student's Test was used in evaluation of all the data before and after the treatment period. All the analyses were done using the SAS statistical package, version 5.18 (SAS Institute Inc., Cary, N.C.). Probabilities less than 0.05 were considered significant.

RESULTS AND COMMENT

As clearly evident from Fig. 5 the chitin nanofibrils (CN), used in all the formulations as skin penetration enhancer, seem to increase the antioxidant efficacy of the active compounds selected to reduce oxidative stress. Recently it was demonstrated that CN is capable of penetrating throughout the skin layers in conjunction with other active compounds, facilitating their penetration power (13).

Probably this polyglucoside compound drives and activates some specific biochemical processes such as desquamation, modulation of extracellular lipid lamellae and sebum secretion, facilitating the possible interactions between the emulsion, the selected active compounds and the skin.

It may also lead to a reversible deformation in the bilayer structure that allows the creation of various types of openings in the skin bilayers. These openings can trigger a thermodynamic alteration within the lipid domains leading to increased lipid fluidity or creation of actual microscopically visible pores.

This of course may explain the activity observed in vivo. On the other hand the increased activity in vitro, may be explained by a reversible interference by CN with the cell’s metabolic activity. As it is known the papillary portion of the dermis contains a high amount of collagen fibers required to give firmness and elasticity to the skin.

Fibroblasts are assigned to the continuous production of collagen while consuming ATP as energy.

Then the effects some antioxidant/immunomodulating compounds mixed with CN would have on the growth of fibroblast cultures and collagen production were studied. As evident in fact, in Fig. 1, the in vitro activity of fibroblasts is normally increased by the use of the antioxidant lutein/melatonin and the immunomodulant ectoin. Also fibroblast growth is increased when CN is added to this mixture of active compounds, with the consequent increase in collagen production. (Fig. 3).

The same results are obtained when the enzymatic ATP activity after UV-irradiation was examined (Fig.2). In fact, when fibroblasts are irradiated by UV, oxidative reactions occur affecting both oxygen-sensitive substances and ATP activity with the result of obtaining a decrease of the ATP content and an increase of lipid peroxides. The in vivo studies have confirmed these data.

The antioxidant/immunomodulant compounds used have demonstrated, therefore, an interesting hydrating (Fig.9) and whitening activity (Fig.4), normalizing also the surface skin lipids (Fig. 10) of subjects suffering from a particular photoaged dry skin.

What is interesting to underline is the capacity the emulsion has, to simultaneously reduce the TEWL (Fig.8) and the lipid peroxides (Fig.7), thus demonstrating an interesting global antiaging activity.

Last but not least, the right combination of these antioxidant/immunomodulant compounds carried by CN have demonstrated interesting anti-inflammatory effects both on people affected by atopy and on normal subjects. The formulation, in fact, easily decreased the elevated interleukin-8 (IL-8) of some volunteers affected by atopy (Fig.6), but also seemed to be able to decrease sub-clinical erythema due, for example, to UV exposure (Fig.11). It was demonstrated therefore, that this formulation has the possibility to
decrease the microcirculation, while reinforcing the skin’s vascular system, and highly decreasing sub-clinical erythema, when applied before UV exposure. Moreover its activity is increased about 20% by the addition of CN.

If the formulation is applied just after the UV irradiation, the reduction in sub-clinical erythema is still significant, but not as great as when used in the pre-irradiation period.

This difference of activity supports the conclusion that CN and the active ingredients used in the formulation, are all powerful free radical scavengers and, therefore, more active at the time of free radical production. For this reason also the right combination of melatonin, lutein and ectoin carried and empowered by the use of the chitin nanofibrils can help to prevent the long-term adverse effects of solar radiation and environmental pollution on the skin, including photo-aging, wrinkling and sagging.

**FINAL CONCLUSION**

The significance of these findings, together with the numerous recent reports of the bio-activity of chitin nanofibrils (CN) indicate this natural polyglucoside as a very promising active carrier for innovative cosmetics, diet supplements and bio-materials.

The level of fluorescence detected in the different skin layers (Fig.5) has demonstrated that CN may be used as a penetration enhancer of different active compounds also. The level of redox activity detected, in fact, on the skin treated with antioxidant compounds (product A and B) is further proof of this activity (20,24).

The CN nanosize, composed of innumerable nanoparticles, has interesting film-forming properties, efficient in delivering enhanced moisturization by reducing TEWL. Moreover, their capacity to easily entrap active ingredients, gives them the possibility to diffuse gradually into the skin, from the site of application. The CN’s good stability at different pH (from 0 to 12) and temperatures (from 0 to 240°), its ability to stimulate collagen synthesis, to protect the ATP production and its inherently good antireactive and wound healing properties, when associated or not with other natural active compounds, or used as penetration enhancer, opens up a great number of possibilities to create future innovative cosmeceuticals, nutraceuticals, medical devices and health fibers. Other studies showed CN able to improve the SPF values of sun-protective emulsions by its booster activity, water-resistance and hydrophobic character, increasing also skin hydration as moisture absorber (32-36).

Moreover, it seems able to reinforce the extracellular matrix (ECM) helping in promoting and improving skin firmness and elasticity reducing the appearance of wrinkles. Finally, CN has bacteria inhibiting properties, and promotes also longer perfume endurance through a tighter adherence between skin and perfume, as well as improves dermatological compatibility of preservation agents, bacterial and anti perspirant agents, used, for example, in deodorant formulas (36, 37).
References


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