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*Article*

# Chitin-Hyaluronan Nanoparticles: A Multifunctional Carrier to Deliver Anti-Aging Active Ingredients through the Skin †

**Pierfrancesco Morganti [1,2,3,*], Marco Palombo [4], Galina Tishchenko [5], Vladimir E. Yudin [6], Fabrizio Guarneri [7], Maria Cardillo [3], Paola Del Ciotto [3], Francesco Carezzi [3], Gianluca Morganti [3] and Giuseppe Fabrizi [8]**

1 Skin Pharmacology, Dermatology Department, 2nd University of Naples, 80138 Naples, Italy
2 Dermatology Department, China Medical University, Shenyang 110001, China
3 Research & Development, Centre of Nanoscience, Mavi Sud, s.r.l, 04011 Aprilia (Lt), Italy; E-Mails: a.cardillo@libero.it (M.C.); paola.delciotto@mavicosmetics.it (P.D.C.); francesco.carezzi@mavicosmetics.it (F.C.); gianluca.morganti@mavicosmetics.it (G.M.)
4 Department of Plastic, Reconstructive and Aesthetic Surgery, CTO (Centro Traumatologico Ortopedico) Hospital, 00142 Rome, Italy; E-Mail: palombom@virgilio.it
5 Institute of Macromolecular Chemistry, AS CR (Academy of Sciences of the Czech Republic), v.v.i., Prague 6, 162 06, Czech Republic; E-Mail: tiscenko@imc.cas.cz
6 Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg 195251, Russia; E-Mail: yudin@hq.macro.ru
7 Dermatological Department, University of Messina, 98100 Messina, Italy; E-Mail: f.guarneri@tiscali.it
8 Dermatological Department, University of Parma, 43100 Parma, Italy; E-Mail: Giuseppe.Fabrizi@unipr.it

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* Author to whom correspondence should be addressed; E-Mail: pierfrancesco.morganti@mavicosmetics.it or info@mavicosmetics.it; Tel.: +39-6-9286-2634; Fax: +39-6-9281-523.

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**Abstract:** The paper describes the process to produce Chitin Nanofibril-Hyaluronan nanoparticles (CN-HA), showing their ability to easily load active ingredients, facilitate penetration through the skin layers, and increase their effectiveness and safety as an

anti-aging agent. Size and characterization of CN-HA nanoparticles were determined by Scanning Electron Microscopy (SEM) and Zetasizer, while encapsulation efficiency and loading capacity of the entrapped ingredients were controlled by chromatographic and spectrophotometric methods. Safeness was evidenced on fibroblasts and keratinocytes culture viability by the MTT (Methylthiazol) assay; anti-aging activity was evaluated *in vitro* measuring antioxidant capacity, anti-collagenase activity, and metalloproteinase and pro-inflammatory release; efficacy was shown *in vivo* by a double-blind vehicle-controlled study for 60 days on 60 women affected by photo-aging. In addition, the CN-HA nanoparticles have shown interesting possibility to be used as active ingredients, for designing and making advanced medication by the electrospinning technology, as well as to produce transparent films for food packaging, by the casting method, and can be used also in their dry form as tissues or films without adding preservatives. These unusual CN-HA nanoparticles obtained from the use of raw materials of waste origin may offer an unprecedented occasion for making innovative products, ameliorating the quality of life, reducing pollution and safeguarding the environment's integrity.

**Keywords:** chitin nanofibrils; skin aging emulsions; innovative beauty masks; biopolymers; skin delivery; electrospinning; casting technology; fishery by-products; biomass waste

## 1. Introduction

The multi-functionality and safeness of Chitin Nanofibril-Hyaluronan (CN-HA) micro/nanoparticles, used as cosmetic emulsion because of their 100% biodegradability and skin friendly activity, the targeted delivery capacity to overcome the skin barrier acting as an anti-aging carrier, and the possibility to also be used in their dry form as tissues with structural similarities to native extra cellular matrix (ECM) and as transparent films free of preservatives, offer unprecedented occasion for making innovative products to ameliorate the quality of life, and reduce pollution and greenhouse gas emissions.

The use of these unusual nanoparticles, which are in fact obtained from the raw material of waste, safeguards environment integrity by addressing biodiversity loss, which is already estimated to cost the EU (European Union) around 450 billion Euro per year.

Progress in nanotechnology have given rise to the possibility to design, produce and engineer delivery systems as nanoparticles and smart nanoemulsions maximizing the effectiveness of the ingredient(s) entrapped or encapsulated [1–4]. The physicochemical properties of these nanoparticles can be engineered at the molecular level [5,6], while their shape, size, and electrical charge, as well as the surface density of their targeting ligands for specific applications, can be easily controlled [7–10]. Thus, it is possible to develop a nanocarrier that, loaded with active ingredients, may accumulate selected compounds in certain skin areas owing to its physicochemical and distributing properties. In this way, these effective ingredients may be delivered into different skin layers and released in a controlled manner for optimal dosing [11]. Tuning of the physicochemical properties of nanoparticles to achieve site-specific accumulation is, therefore, an attractive approach that takes advantage of

physiological defects and cellular interactions with nanomaterials used, for example, to design anti-aging cosmetic products or emulsions, reparative non-woven tissues and films as beauty masks that, preservative free, may have antiseptic or anti-inflammatory activities depending on the ingredients entrapped into the nano/micro fibers. These smart and innovative beauty masks should be used for helping, for example, people affected by acne. Because of their lack of toxicity, interesting electrochemical properties, biodegradability, biocompatibility, and high mucoadhesive properties, many natural polymers, such as chitin nanofibrils obtained from fishery/crustacean waste [12–15], and/or some lignocellulosic compounds, extracted from plant biomass [16,17], have been used as nanoparticle delivery systems [18–20]. Among these, Chitin Nanofibrils (CNs), being prevalently covered by positive electrical charges, have shown the possibility to form block-copolymeric nanoparticles (BCC) when directly in contact in water suspension with electronegatively charged polymers such as Hyaluronan (HA) or other negative natural compounds. It is interesting to underline that these nanoparticles can easily entrap in the same way differently sized active ingredients that are hydro or liposoluble [21]. The same entrapping activity has been shown by the use of CN that, mixed with other natural polymers, has been treated by electrospinning technology or by casting technology to obtain respectively porous matrixes as scaffolds for tissue-engineering purposes and beauty masks [22], as well as thin films [23].

The aim of this paper is to report results of different studies characterizing and controlling the bioavailability and safety of block-copolymeric CN-HA nanoparticles, entrapping lutein and other active ingredients, just to underline their effectiveness as compounds of anti-aging cosmetic emulsions. In addition, non-woven biomimetic tissues and biodegradable films made by the use of CN and other natural polymers will be shown as first results of the EU research projects *Chitofarma* and *n-Chitopack*.

## 2. Experimental Section

### Materials and Methods

#### Materials

Lutein crystals (99%) were purchased from Kemin (Kemin Food, Des Moines, IA, USA), Hyaluronic Acid (HA) from Kibun (Kibun Food Chemifa Co., Tokyo, Japan), Chitin and Chitosan from Primex (Siglufjordur, Iceland), lignocellulosic polymer from Compagnie Industrielle de la Matière Végétale (Labège, France) while Chitin nanofibrils (CN) block-copolymeric nanoparticles and nanoemulsions were purchased from MAVI sud S.r.l. (Aprilia, Italy).

#### Methods

Nanoparticles preparation and characterization: *in vitro* and *in vivo* activity. According to gelation method (Boochemal *et al.* [24] modified from our group [25], it was possible to obtain block-copolymeric CN-HA micro/nanoparticles slowly dropping the acidic suspension of CN (2% w/v) into a stabilized suspension of HA (2% w/v) by a syringe with a 30-gauge needle under high speed and constant stirring, as reported in Figure 1.

The HA water suspension contained a stabilized hydrophilic surfactant, while CN contained both a lipophilic stabilizer and lutein as example of an active ingredient (2 mg% w/v). The obtained morphological characterization of the micro/nano lamellar complexes entrapping different active ingredients, purified by centrifugation, re-suspended in distilled water, treated by high-pressure homogenizer, and atomized in a stream hot air, have been controlled by Scanning Electron Microscopy (SEM) (Philips XL20, Amsterdam, The Netherlands) [26] as shown in Figure 2.

The medium size of the lutein-loaded nanoparticle was controlled by a Zetasizer (NanoZS Model 3600—Malvern Instruments, Worcestershire, UK), while its release was measured by a dissolution apparatus (Distek 2100 B) and controlled by HPLC (High Performance Liquid Chromatography, Varian 9012, Varian Associates Inc., Palo Alto, CA, USA). Both the encapsulation efficiency of CN and the loading capacity of the entrapped active ingredients, compared with chitosan and amorphous commercial chitin, were determined by gel filtration chromatography, and analyzed by spectrophotometer and HPLC, as reported in Table 1 [24,25].

**Table 1.** Nanoparticles yield, Lutein loading content and entrapment efficiency of different kind of chitin and chitosan complexed with hyaluronic acid.

| Polymer | Nanoparticle yield (%) | Lutein loading content (%) | Entrapment efficacy (%) | Particle mean size (%) |
|---|---|---|---|---|
| Chitosan-HA-Lutein | 33 ± 9 | 10 ± 3 | 32 ± 5 | 458 ± 14 |
| Amorphous Chitin-HA- Lutein | 31 ± 10 | 18 ± 3 | 40 ± 5 | 355 ± 13 |
| Crystal-Chitin HA (CN) Lutein | 42 ± 9 | 35 ± 3 | 66 ± 6 | 185 ± 13 |

Note: All measurements were performed in triplicate; CN = Chitin nanofibrils; HA = Hyaluronic acid.

**Figure 1.** The gelation method between Chitin nanofibrils (CN) and Hyaluronan (HA) nanoparticles.

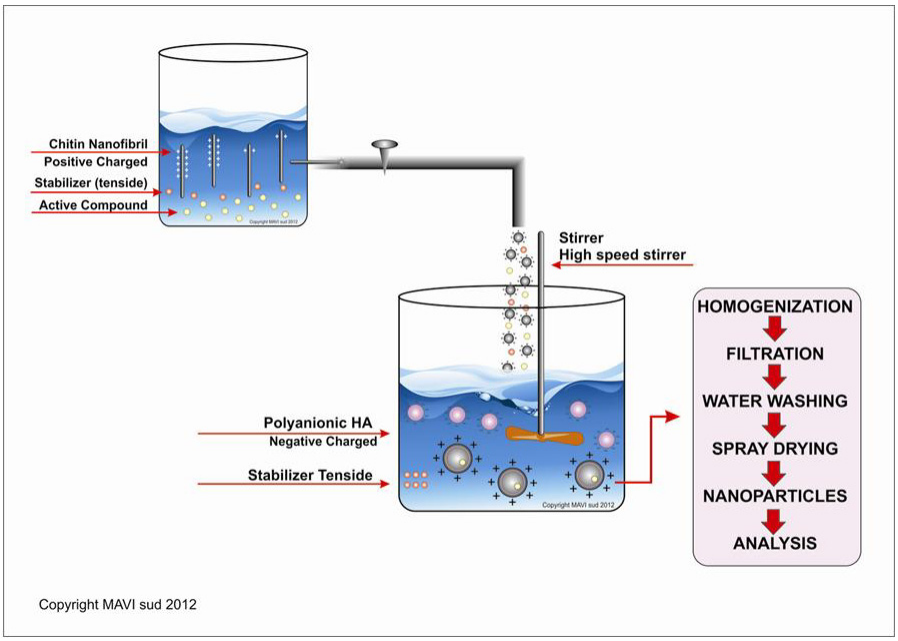
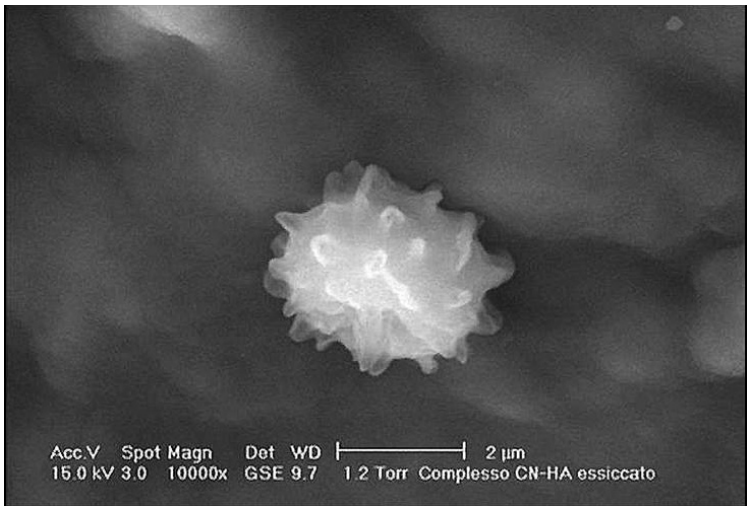


**Figure 2.** Nanoparticles of CN-HA at SEM.




Moreover, the cytotoxicity of the different nanoparticles was performed *in vitro* measuring the keratinocyte and fibroblast viability by the MTT assay, normally utilized by our group (Figures 3 and 4) [25–27].

In addition, the supposed anti-aging activity was controlled both *in vivo* and *in vitro*, evaluating the antioxidant and the anti-inflammatory activity, as well as the balancing effect on the anticollagenase and the metalloproteinase release. It was controlled the emulsion efficacy *in vivo* by a double-blind vehicle-controlled study for a period of 60 days, on a group of 60 women affected by photo-aging [27]. Finally, the skin penetration was controlled *in vivo* by the tape stripping method (D-Squame, Cu Derm, Dallas, TX, USA) and the scrub technique, applying the cream (2 mg/mL) in a random manner for one month of treatment [27].

**Figure 3.** Effect of Chitin Nanofibril-Hyaluronan nanoparticles on the viability of keratinocytes. All *p* values are not significant as the control and the groups.

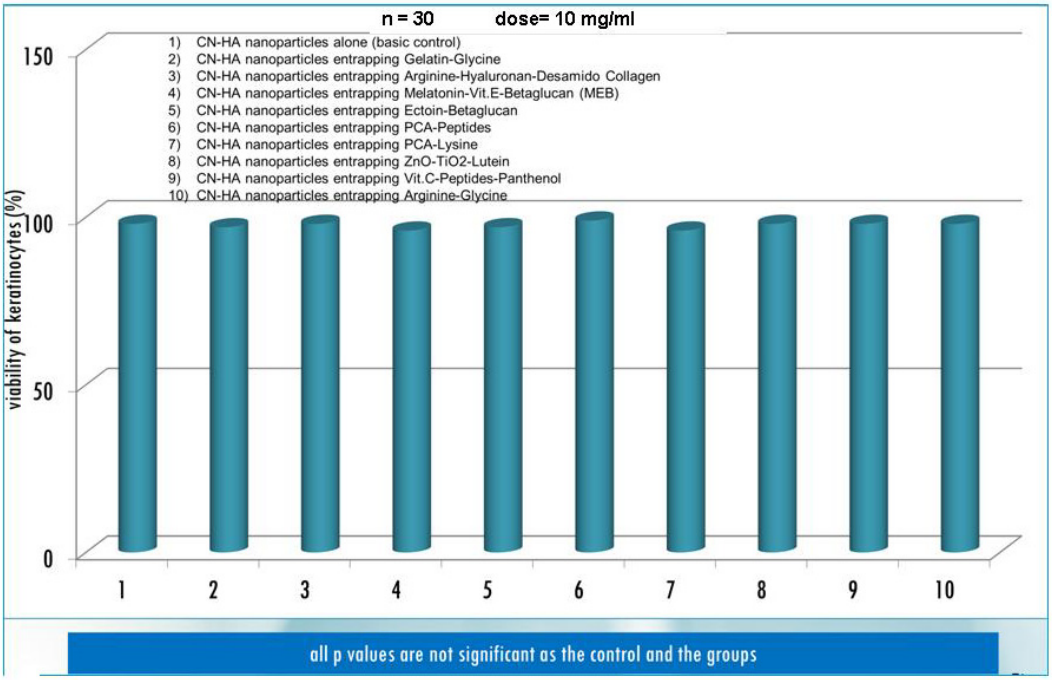
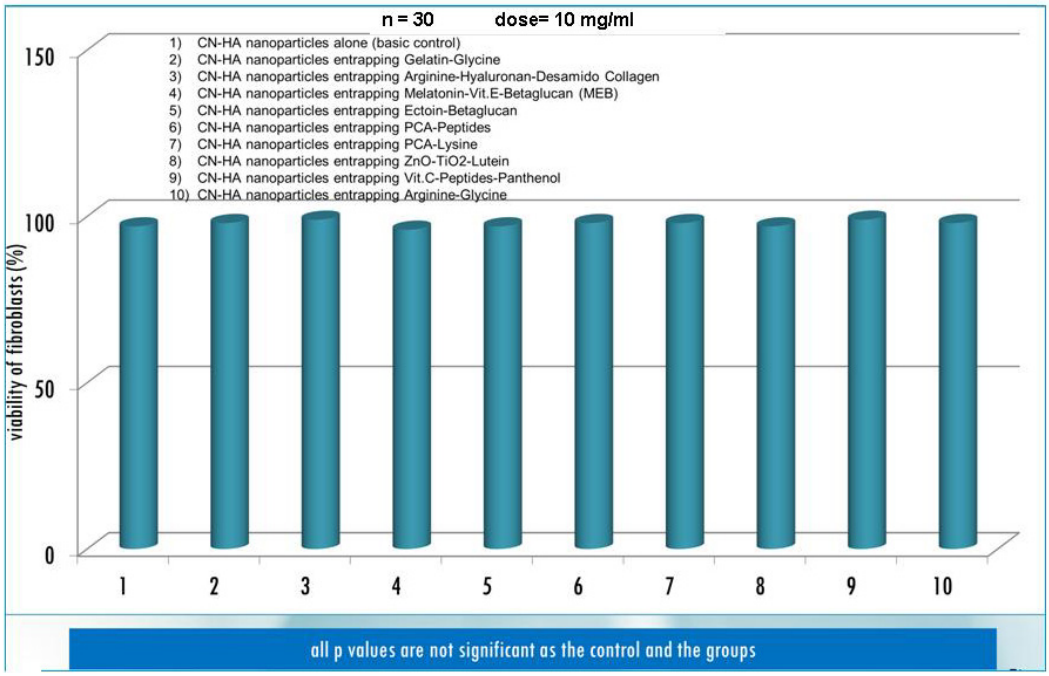


**Figure 4.** Effect of Chitin Nanofibril-Hyaluronan nanoparticles on the viability of fibroblasts. All *p* values are not significant as the control and the groups.




## 3. Results and Discussion

Nanocosmetology may be defined as the cosmetic intervention at the molecular scale to repair and control human biological systems of skin, aged or affected by minor disorders, such as acne and xerosis. Basic nanostructured materials, engineered enzymes and the many other products of biotechnology will most probably become essential elements in some areas of medical and biological applications to design innovative cosmetics and a new generation of advanced beauty biomasks and medications [28,29]. However, the full promise of nanocosmetology is yet to be defined, because it will depend on the development of new technologies necessary for a more precise control of the effectiveness and safeness of the ingredients used, as well as of the obtained final products.

In this study, we try to define this fascinating area of research suggesting the use of Chitin Nanofibrils (CN) as a biomimetic natural innovative carrier, reporting some technologies used to control the effectiveness and safeness of anti-aging cosmetic products based on this nano-ingredient [30,31].

Thus, by the use of the gelation method [24,25], we bonded together the electropositive CN with the electronegative hyaluronic acid, obtaining block-copolymeric nanoparticles (BCC) in the size range of 40–200 nm (Figure 2). Naturally, the dimension of BCC depends on the way of operating the carrier (emulsion) as well as on the active ingredients' entrapment [26,32].

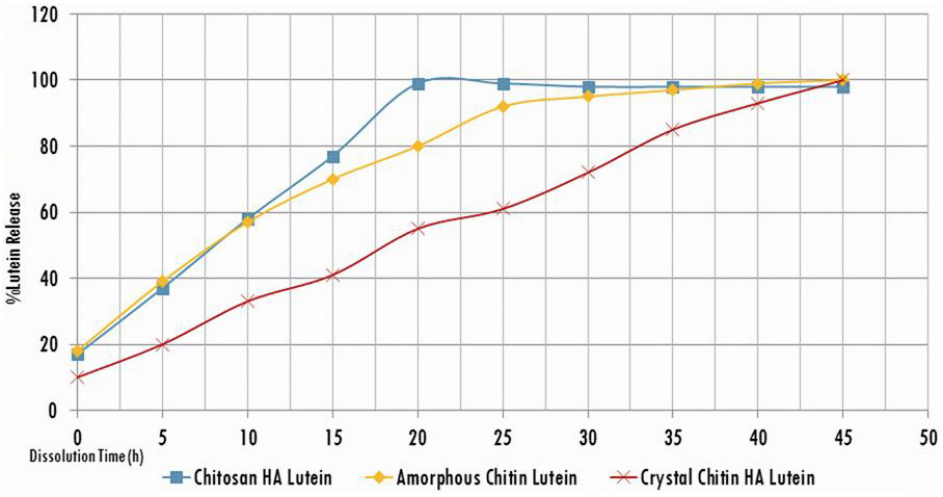
Characterization and size of nanoparticles, their release profile and degradation, loading content and related entrapment, essential for a thorough understanding of their properties, efficacy and degradation behaviour, are reported on Table 1 and Figure 2, regarding the entrapped lutein [26,32]. Molecular weight of the CN-polymer was used together with the encapsulated ingredient influence—in fact, the nanoparticle size, entrapping efficiency, and degradation rate of the nanoparticles—hence affecting the release rate of active ingredient(s). These phenomena have been also underlined by different authors for the use, for example, of polylactide-*co*-glycolide and other polymers (PLGA) [33–36].

However, degradation, as well as active ingredient release, can be precisely controlled, characterizing in advance the physicochemical properties of the polymer, such as its molecular width, polydispersity index, hydrophobicity, hydrosolubility and crystallinity.

Soon after, the entrapped active ingredients can be released in a controlled manner due to their diffusion through the polymeric matrix, or be triggered in response to environmental stimuli, or released in the course of enzymatic degradation.

According to our used method [25], the release of lutein entrapped into CN-HA was measured by the dissolution apparatus Distek 2100 B equipped with an autosampler and assayed by HPLC at 490 nm. The results obtained are reported in Figure 5 [25].

**Figure 5.** Release profile for lutein from chitin/chitosan nanoparticles.




Moreover, as reported in Figures 6 and 7, it is interesting to underline how the *in vitro* results have been confirmed from the *in vivo* studies, showing a good skin penetration and bioavailability of lutein recovered at the level of different stratum corneum layers (SC) at different times, depending on the size and charge of the nanoparticles designed and, of course, on the method used, such as the stripping and desquamation techniques measuring the skin turnover [37,38].

Because of the crystallinity of CN and their methodology of production, these nanoparticles, covered by positive surface charges, seem to have an interesting ability to disturb the tight lamellar layers of the SC, enabling a better diffusion of entrapped active compounds through the dipper's skin layers as well. On the contrary, when the nanoparticle surface is covered by negative charges, the active ingredients remain at the level of the outermost skin (Figure 8).

As a consequence of the positive CN-HA nanoparticles obtained, it was possible to modify the quantity and quality of SC lipids, repairing the disrupted skin barrier of subjects affected by photo-aged skin (Figure 9) [39] or acne (Figure 10) [40], as well as to re-balance skin hydration from inside and outside (Figure 11) and skin elasticity (Figure 12) of subjects affected by photo-aging [27,41]. It is also interesting to underline that the nanoparticles, entrapping the antioxidant complex Melatonin-vit E-Betaglucan (MEB), have shown *in vitro* the best activity in increasing collagen production (Figure 13) at the level of fibroblasts cultures, contemporarily inhibiting the collagenase activity (Figure 14) and the MMP1 (Metallo Proteinases1) (Figure 15) and IL-8 release (Figure 16) compared to other active ingredients used [27,31].

**Figure 6.** Lutein recovery of different stripped skin layers after one month of treatment by CN-nanoparticles applied on left or right forearm. All $p$ values are significant ($p < 0.005$) as control and as groups; * = not significant.

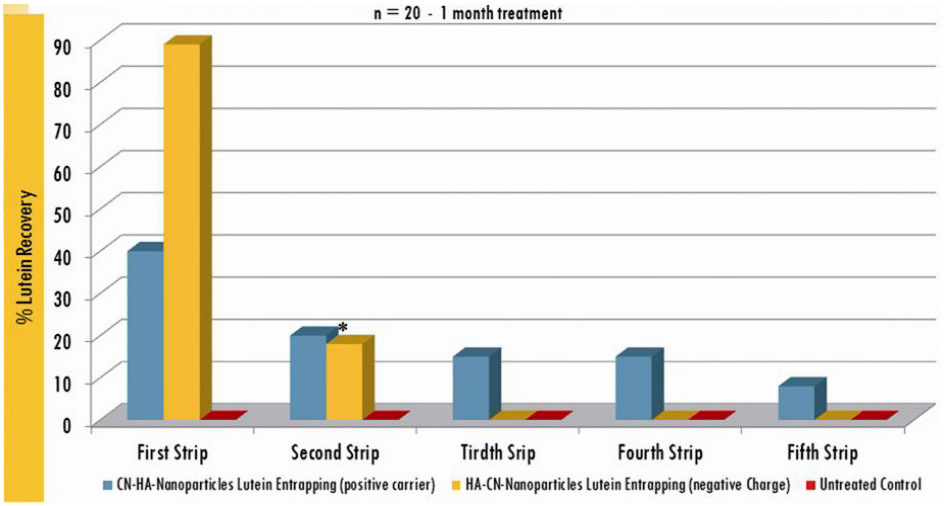



**Figure 7.** Lutein recovery of corneocytes removed from forearm by forced scaling at different times and days of treatment by nanoparticles entrapping lutein. All $p$ values are significant ($p < 0.005$) as control and as each other groups.

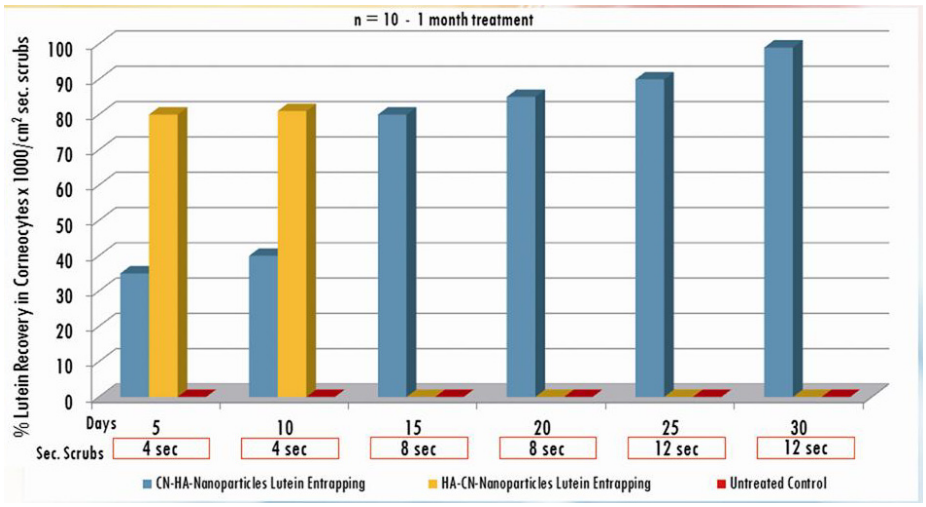



**Figure 8.** CN-HA has the capacity to disturb the stratum corneum lamellae organization, increasing the skin penetrability of the active ingredients entrapped.

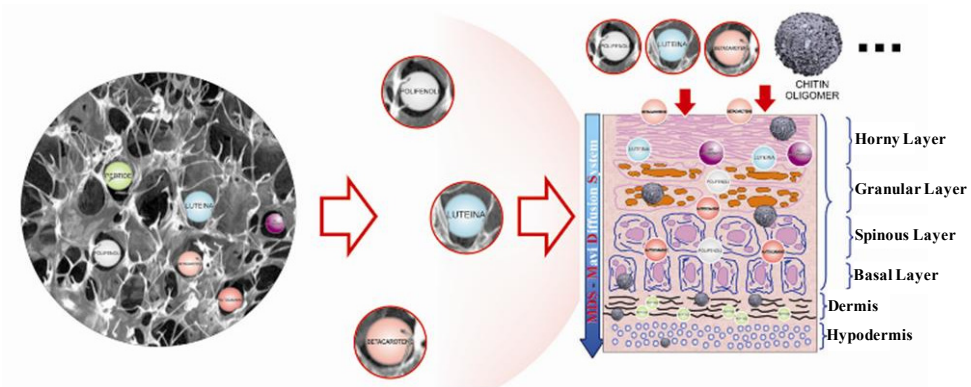


**Figure 9.** Activity of chitin nanofibrils alone or complexed with antioxidant and immunomodulant ingredients (active cream) as a vehicle on superficial skin lipids of the skin of photo-aged women. All $p$ values are highly significant as control and as each other groups ($p < 0.005$).

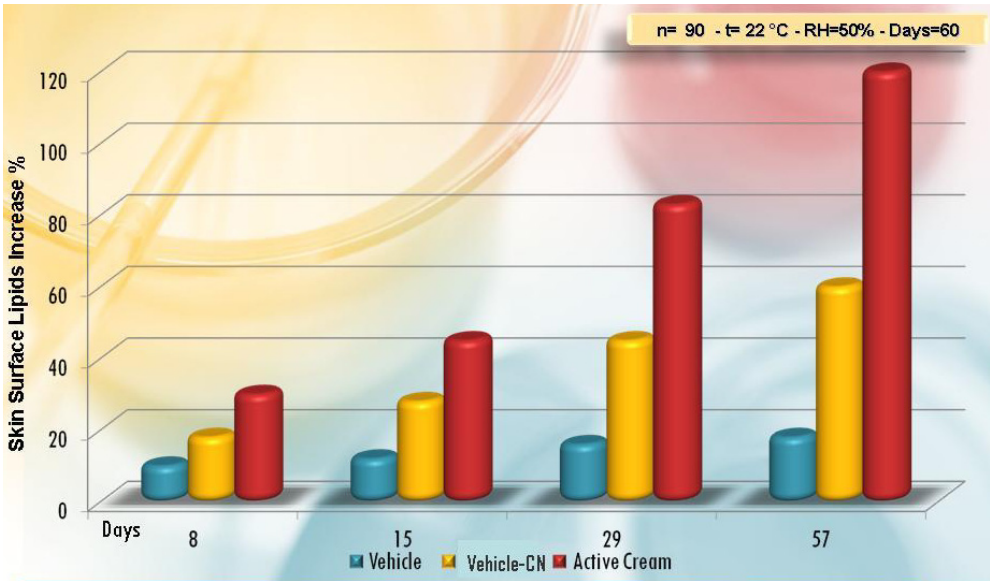
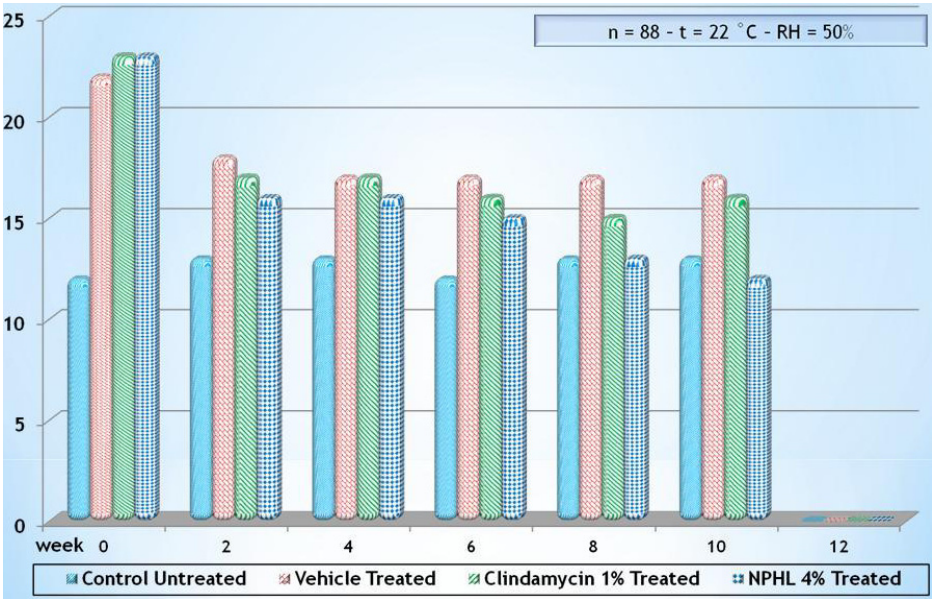



**Figure 10.** Transepidermal water loss (TEWL) of acne affected patients treated by 4%-nicotinamide phosphatidylcholine linoleic acid rich emulsion *vs.* 1%-clindamycin phosphate and the vehicle. All $p$ values are significant as groups and as baseline ($p < 0.05$) clindamycin *vs.* NPHL (Niacinamide-Phosphatidylcholine) ($p < 0.05$).




Moreover, it has been shown that CN-HA size and its physicochemical characteristics can enhance the cellular uptake: the smaller the particle, especially when positively charged, the easier it can be delivered to cells, acting as cell signalling [42].

Topical use of antioxidant compounds seems, in fact, useful to remove free radicals in excess, preventing also the consequential increased production of inflammatory cytokines and MMPs. The

efficacy shown by CN-HA nanoparticles is probably due to the double activity of Hyaluronan and Chitin (polymer of glucosamine and acetyl glucosamine), capable of creating a favourable enzymatic environment for skin turnover. These particular conditions, further reinforced by the activity of the antioxidant ingredients entrapped, facilitate the skin's cell migration and function, sustaining the synthesis of both ECM and fiber-macromolecules, neutralizing the oxidative activity of free radicals in excess.

**Figure 11.** Skin hydration of photo-aged healthy subjects treated topically and/or orally by antioxidant compounds with chitin nanofibrils (% increase *vs.* baseline values). All *p* values are highly significant ($p < 0.005$) as baseline, placebo and significant ($p < 0.05$) as to groups.

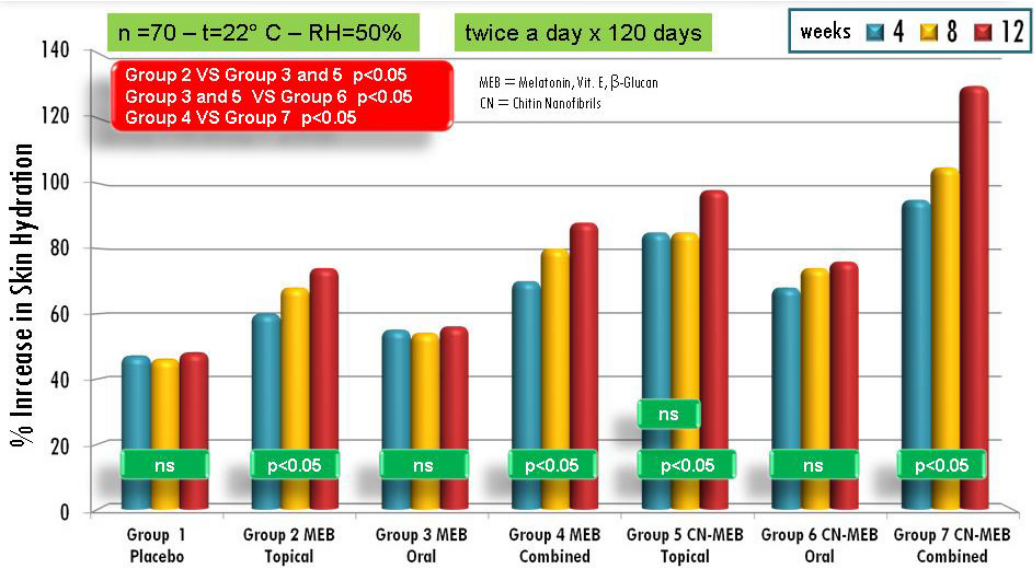



**Figure 12.** Skin elasticity of photo-aged healthy subjects treated topically and/or orally by antioxidant compounds complexed with chitin nanofibrils (% increase *vs.* baseline values). All *p* values are highly significant ($p < 0.005$) as baseline, placebo and significant ($p < 0.05$) as to groups.

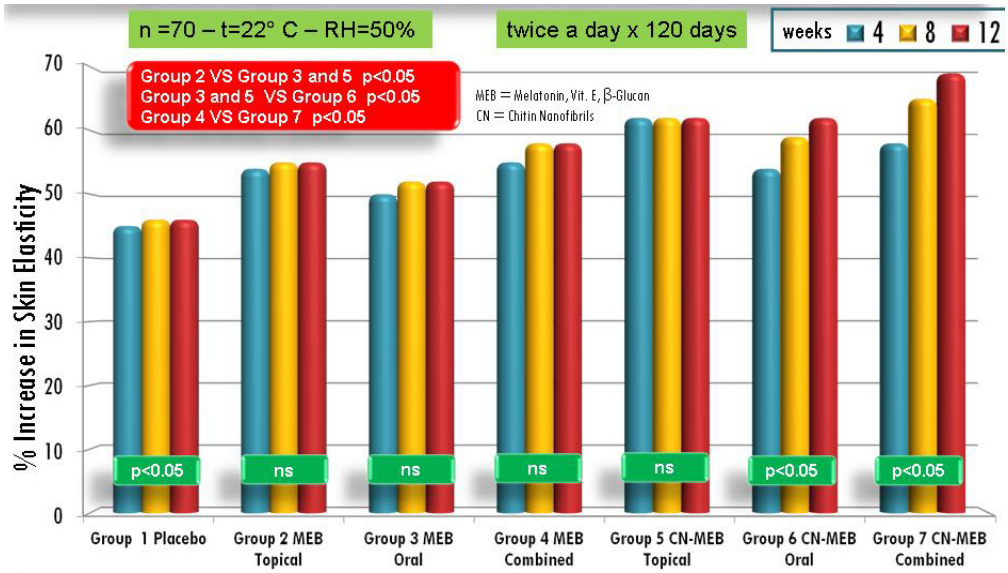


**Figure 13.** Percentage increase of collagen produced by fibroblast cultures added with the liposomial complex phosphatidylcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (ALC) *vs.* untreated control. ALC values *vs.* control highly significant ($p < 0.001$).

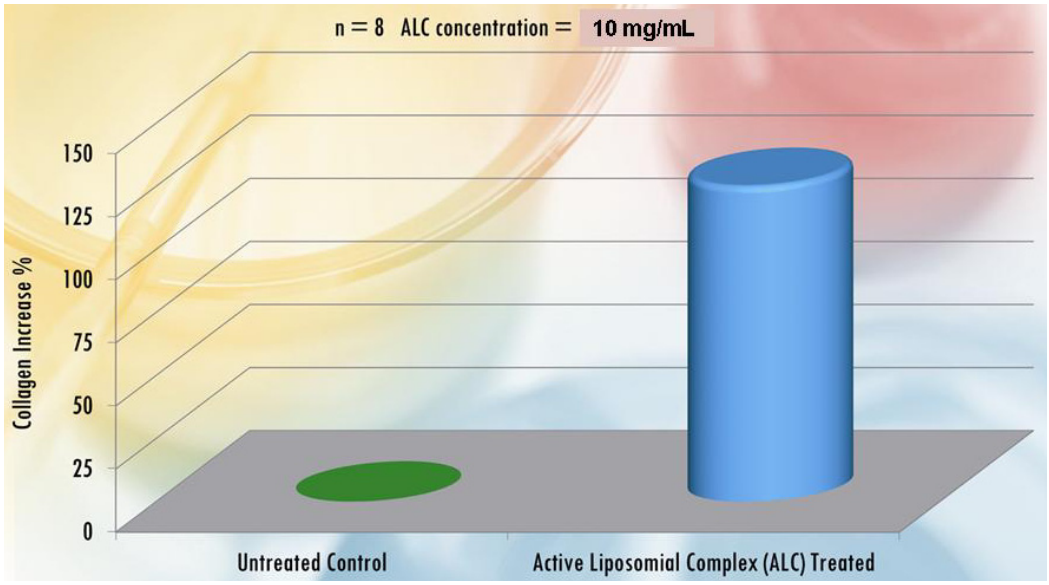



**Figure 14.** Inhibitory activity *vs.* collagen degradation of chitin nanofibrils-hyaluronan nanoparticles entrapping active ingredients in fibroblast culture incubated collagenase enzyme. All $p$ values highly significant ($p < 0.005$) as the control and groups.

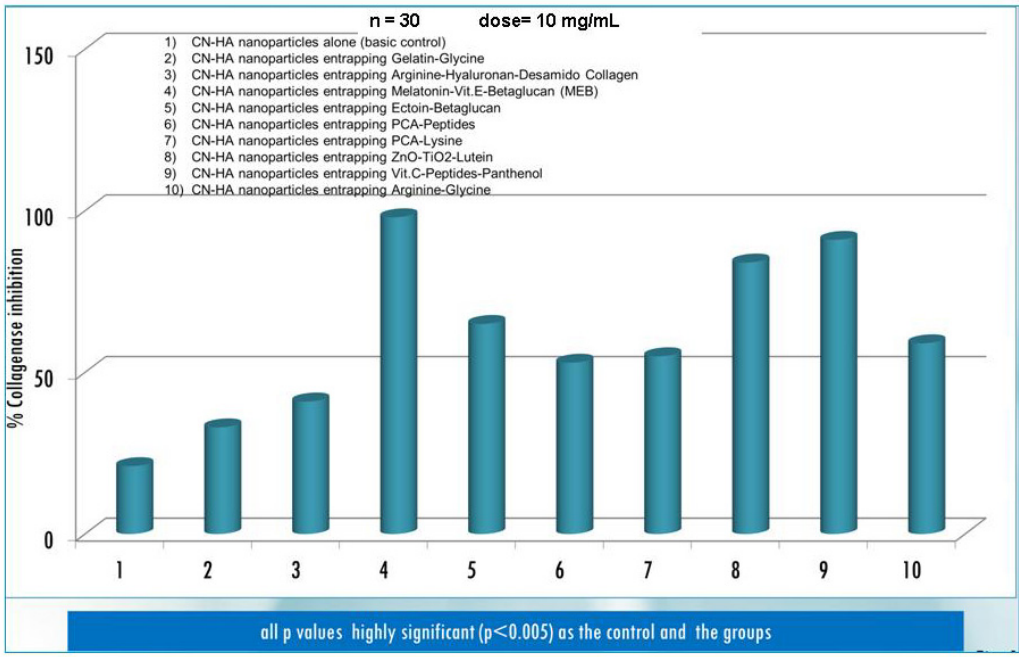


**Figure 15.** MMPl release in aged fibroblasts treated by chitin nanofibril-hyaluronan (CN-HA) entrapping active ingredients. All $p$ values highly significant ($p < 0.005$) as untreated, TGF-β (Tumour Growth Factor-β) treated and as groups.

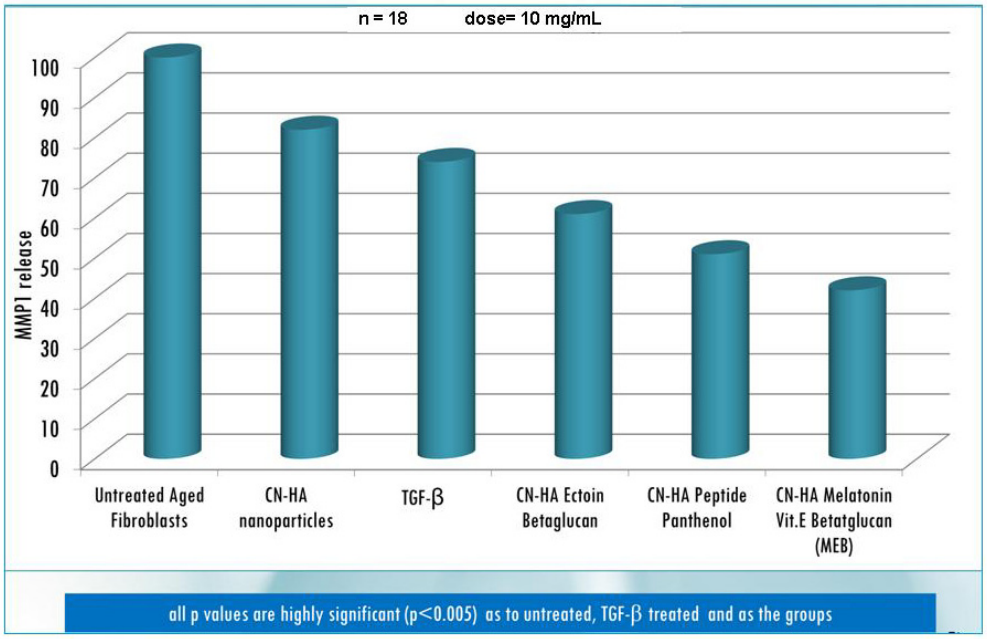
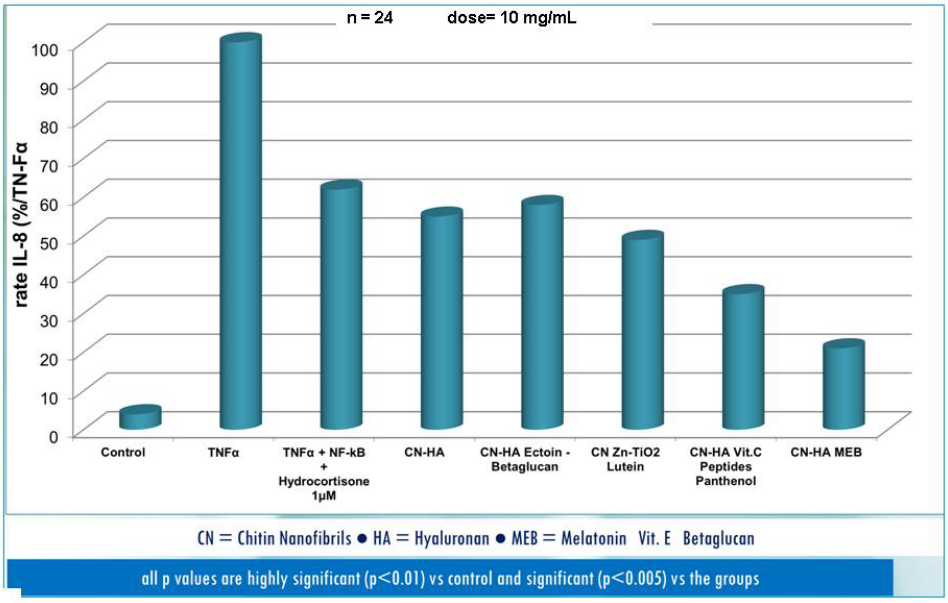



**Figure 16.** Inhibition of IL-8release on TNF-α (Tumour Necrosis Factor- α) stimulated human keratinocytes. All $p$ values are highly significant ($p < 0.01$) *vs.* control and significant ($p < 0.005$) *vs.* the groups.




Finally, as previously reported in Figures 3 and 4, all the studied block-copolymeric nanoparticles have shown to be non-toxic on the viability of both keratinocytes and fibroblasts' cultures [40,41]. On the other hand, it is interesting to underline the capacity CN-HA has to increase the UV-screening efficacy of both the inorganic and organic sunscreening agents trapped in a block polymeric structure (Table 2) [43].

**Table 2.** Comparison of SPF and UVA-PF activity of chitin nanofibrils-hyaluronan entrapping carotenoids, ZnO and $TiO_2$. All $p$ values are highly significant as control ($p < 0.005$) and significant as each other groups. Zn = zinc oxide nanoparticles; $TiO_2$ = titanium dioxide nanoparticles; CN = chitin nanofibrils; HA = hyaluronic acid.

| Active compounds | SPF (Sun Protection Factor) | UVA-PF (UVA Protection Factor) |
|---|---|---|
| Zn-$TiO_2$ Alone (control) | 20 ± 1.8 | 7 ± 0.8 |
| Zn-$TiO_2$ CH-HA entrapped | 30 ± 2.3 | 10 ± 2 |
| Zn-$TiO_2$ Lutein CH-HA entrapped | 50 ± 3.4 | 21 ± 4 |
| Zn-$TiO_2$ β-Carotene CN-HA entrapped | 40 ± 2.9 | 13 ± 3 |
| Zn-$TiO_2$ Lycopene CN-HA entrapped | 45 ± 2.5 | 20 ± 4 |

By this technique, lutein and lycopene have shown to increase the sunscreen activity of ZnO and $TiO_2$, *versus* both UVA and UVB (Ultraviolet rays long waves A and short waves B), much more than betacarotene. Thus, the CN-HA block polymer seems to acts not only as a carrier but also as an interesting boosting agent [43,44]. Moreover, CN and CN-HA nanoparticles have been transformed, by electrospinning technology, in non-woven tissue to be used as innovative beauty masks and advanced medications.

From the first results the obtained micro/nanofibers have shown, in fact, the ability to form interesting scaffolds that, mimicking the size and arrangement of skin's ECM and native collagen fibers, are able to increase cell proliferation rate, maintain cell phenotype, support differentiation of stem cells, and activate cell-signalling pathways as well (Figure 17) [28,29].

Finally, by the EU n-Chitopack project, using the casting method, we are obtaining food packaging nanocomposite films with bacteriostatic and UV-resistant properties of high performance and environmental compatibility (Figure 18). It is also interesting to underline how low quantity of CN seems able to orient the disposition of the chitosan fibers into the nanocomposite film, leading to an increase in strength and Young modulus of the obtained composite fibers (Figures 19 and 20) [45].

**Figure 17.** Non-woven tissue obtained by electrospinning at SEM.

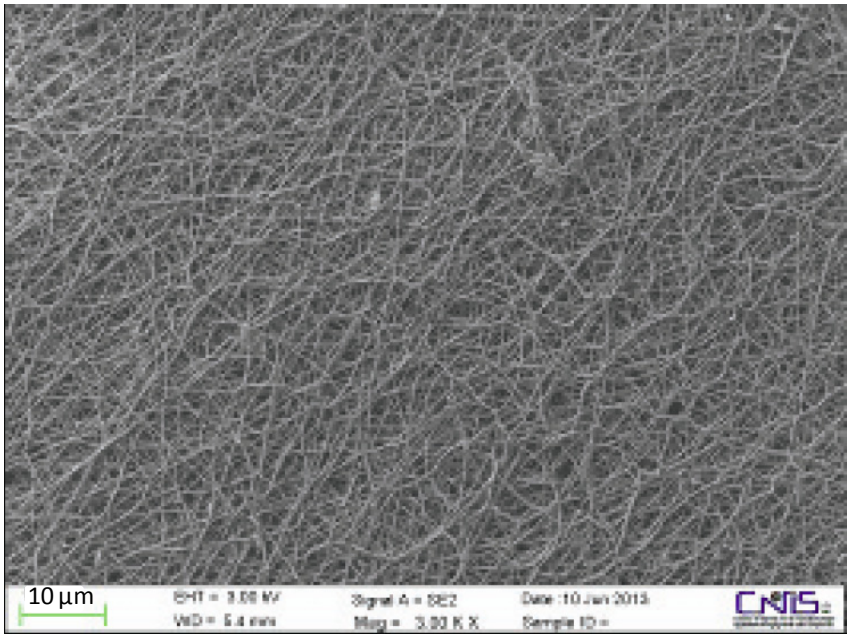


**Figure 18.** Film-tissue obtained by the casting method at SEM and as final product.

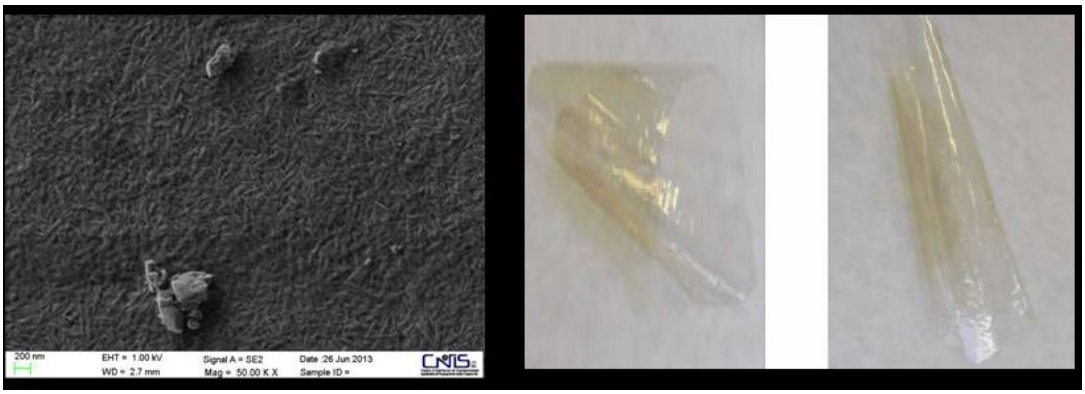


**Figure 19.** The smooth surface of chitosan/CN composite fiber (**a**) shows a regular disposition of CN into its inner structure (**b**). The fiber contains 1 wt% of CN.

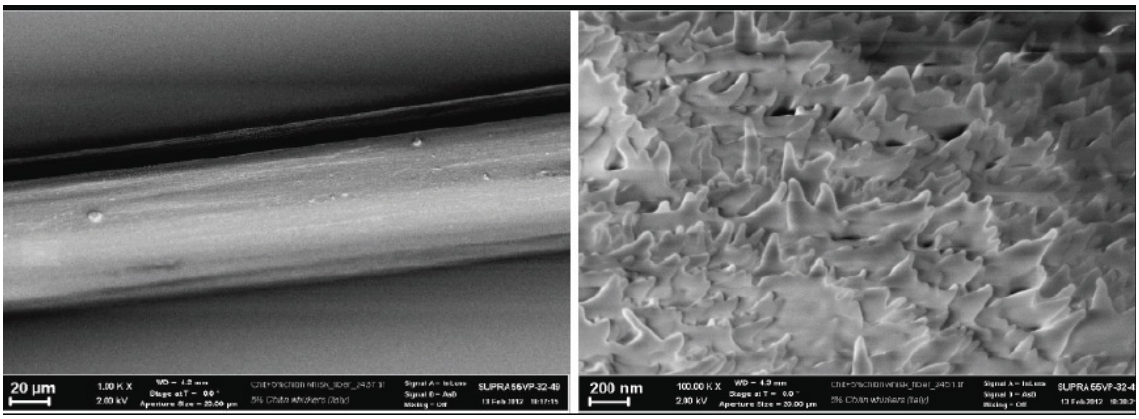
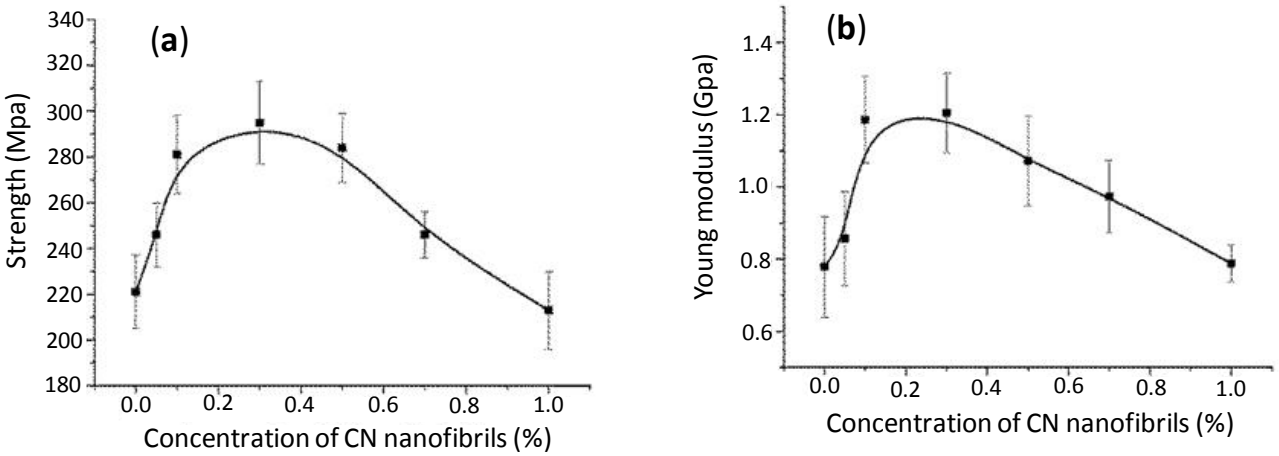



**Figure 20.** Dependences of tensile strength (**a**) and Young modulus (**b**) of the chitosan/CN composite fibers on the content of the chitin nanofibrils.




## 4. Conclusions

Current polymeric nanocarrier technologies, both as nanoparticles/nanoemulsions and non-woven tissues and/or films, have shown remarkable advantages for a drug/cosmetic/food delivery, when compared with conventional emulsions or tissues. Among the other polymers utilized up until today,

CN seems to be a very promising natural ingredient for making innovative anti-aging and skin reparative cosmetic delivery systems, due to its specific characteristics, including good biodegradability and biocompatibility, when taken by oral route as well [41,46–52]. Development of multifunctional CN-HA block-copolymers, or CN complexed with other negatively charged natural polymers, containing specific ingredients for active targeting, will provide new versatile and straightforward approaches such, as the mind-body NICE-activity (Neurological Immune Cutaneous Endocrine systems) [51–55], to improve, for example, the skin aging activity [31,32,41] or accelerate the wound healing processes [56–60]. For further advancement, it will be necessary to focus more research attention on their regulatory status [61] and productive processes, decreasing energy consumption and costs, and increase the use of waste materials to reduce pollution and save humans and the environment.

In conclusion, the application of nano-techniques, at the cellular, molecular and atomic levels, should certainly help in improving understanding of skin biology, as well as in the development of advanced medications or innovative cosmetic products for treating not only aging and photo-aging skin but also minor disorders or mild skin abnormalities.

According to our previous opinion [61], these innovative cosmetic products that show a physiological activity in "suffering skin" also have to be considered under the same cosmetic EU rules as a new class of clinically correct cosmetics, having demonstrated meeting additional *in vitro* and *in vivo* requirements for safety and efficacy.

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## Author Contributions

Pierfrancesco Morganti has coordinated the whole works done. Francesco Carezzi and Paola Del Ciotto have controlled the stability and the safeness of all the studied formulations. Galina Tishchenko and Vladimir E Yudin have contributed to control and define the chemico-physical activity of the Chitin Nanofibrils films. Giuseppe Fabrizi, Fabrizio Guarneri and Maria Cardillo have studied the clinical activity of the different emulsions made by the Chitin Nanofibrils technology.

## Conflicts of Interest

Pierfrancesco Morganti is also the R&D Director of the Nanoscience Centre, Mavi Sud s.r.l., Francesco Carezzi and Paola Del Ciotto work at Nanoscience Centre, Mavi Sud s.r.l., Italy.

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