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Delivering retinol efficiently

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1. Introduction

Retinol is one of the most common cosmetic actives, widely used to manage skin ageing and photo-related cutaneous damage. It is a gold standard that stimulates cell turnover, cell defense and ECM (extracellular matrix) macromolecule production to smooth wrinkles, to modulate skin pigmentation and to reinforce skin barrier function and immunity [1]. Recent research highlighted benefits in hair growth emphasizing the multifunctionality and potency of this cosmetic active.

Retinol was first isolated in 1930s but uneasy to study as very unstable. Retinol rapidly degrades at ambient temperature, after exposure to air, sunlight or ultraviolet rays, rendering its handling challenging and limited. As a side note, retinol is however less photolabile than retinyl esters [2]. Retinoid degradation produces radical intermediates
giving an intense yellow colour to cosmetic formulations and its instability can potentially lead to phase separation of cosmetic preparations. The degradation also affects the efficacy of the formulation as the concentration in active is diminished [3]. Despite their outstanding skin benefits, the skin irritation potential, poor stability and formulation challenges of retinoids feed the cosmetics industry's demand for a more stable, well-tolerated and effective form of vitamin A.

Lipid capsules are delivery systems based on solid lipids surrounding an oil core in which the active is dissolved. This system presents different benefits as increasing active stability, controlling the release, protecting the active from light, reducing side effects and increasing the efficacy. Cutaneous use of lipid nanoparticles presents several advantages, such as the chemical protection of the incorporated substances, allowing the skin application of labile molecules that are difficult to transport in traditional semi-solid formulations; improved active bioavailability, related to the possibility of modulating molecules release, promoting their skin penetration and retention. The latter has been explained by the lipid nanoparticles easiness to adhere to the *stratum corneum*, and the capability of allowing encapsulated molecules reaching the deeper skin layers, as described in literature. These properties are related to the lipid nanoparticles physiological lipid composition that can interact with the Stratum Corneum, creating its lipid rearrangement, which eases molecules penetration. Furthermore, published papers demonstrated that the nanoparticles small size also contributes to increase their adhesiveness and surface contact area, promoting the active influx through the skin.

2. Materials and Methods

Retinol content by HPLC

Retinol content was quantified using an HPLC method with C_{18} column (4.6 mm x 150 mm), C_{18} guard column, mobile phase consisted of water:methanol (5:95 v/v), temperature of 35ºC, flow rate of 1.0 mL/min, injection volume 20 µL and run time of 8 min, monitored using UV-Vis

detector at 325 nm. Prior to HPLC-UV analysis, samples were diluted in isopropanol in amber volumetric flask, stirred and sonicate for 10 min and filtered (PVDF, 0.45 µm).

free retinol stability study
Retinol powder (98.5%) was diluted in isopropanol and used for stability studies. Samples were stored at 5°C, RT, 40°C and 50°C and evaluated at 7, 14, 21, 30, 60 and 90 days. Experiment was done in duplicates. At each time point, new samples were opened and quantified by HPLC for retinol content.

Preparation of encapsulated retinol
The system produced is a lipid capsule, composed of a thin layer of wax involving the oily core of retinol and vegetable oil, stabilized by lecithin and a high HLB surfactant. For production, an exclusive and patented process was used, through the formation of submicrometric wax particles. The final concentration of retinol in the formulations was 3% (w/v).

Characterization of the encapsulated system
Appearance, colour and viscosity were visually evaluated. The samples were analysed for the presence of phase separation, precipitates, colour, and heterogeneous aspects by means of verification in transparent glass without any instruments.
Particle size was determined by laser diffraction technique. The formulation was added directly into the wet dispersion unit containing water under agitation. The results were expressed in DV (50) and DV (90).
To determine the actual location of retinol within the capsules and its water dispersing medium were separated via an ultrafiltration/centrifugation method. Samples are placed in tube with a PES membrane (Vivaspin tubes turbo 4, PES membrane 30 kDa cut) and centrifuged at 4000 rpm for 20 min. Subsequent to this treatment, the water phase was collected and free retinol within the separated external water phase was quantified by HPLC. The encapsulation efficiency (EE) is determined using the following equation:
EE=(Ctotal-Cfree)/Ctotal x100; Ctotal is the total amount of initial encapsulated retinol in the sample and Cfree is the amount of retinol found in the external water phase.
The retinol molecule being naturally fluorescent, fluorescence microscopy was used as a visual method to prove retinol encapsulation. Natural fluorescent molecules will appear black under normal visible light and coloured under fluorescent light, while non fluorescent molecules will appear black under both visible and fluorescent light. Placebo was prepared as retinol free capsules and also visualised after mixing with free retinol. Images were analysed for the comparison of fluorescence.

Study of stability of the suspension of nanoparticles
For the stability study, four batches of the nanoparticle suspension were stored in aluminium bottles for 90 days at 50°C, 180 days at 40°C and 365 days at room temperature. At each analysis time, a new 50 g vial was opened for the first time.
All samples were handled under regular laboratory lighting and normal air conditions. No inert gas was used to fill the vials or handle any samples. Samples were characterized as described above.
The retinol molecule sensitivity to sunlight is well documented in the literature and a key drawback of this molecule to be addressed to guarantee acceptable shelf life and performance. A series of sunlight irradiation experiments were carried out to evaluate benefits of encapsulated retinol compared to free active. Multiwell plates were filled with encapsulated or free retinol (10 % active in vegetable oil) and exposed to UV irradiation for 1 hour:
□ UVA = 8.2 J/cm² (lamp power = 2.30 mW/cm²)
□ UVB = 4.3 J/cm² (lamp power = 1.17 mW/cm²)

Study of active release from nanoparticles

8 Franz cells (4 with free retinol and 4 with the nanoparticle suspension) containing the release medium and PVDF 0.1 µm membrane were prepared and placed in a water bath at a controlled temperature of 32°C. The amount added to the membrane was 20% of the saturation concentration to ensure sink conditions. The chosen release medium was 3% tween 80 in water and the saturation concentration of retinol in this medium was 2.98 mg/mL. Samples were taken at selected intervals from 0.5 to 76 h with fresh medium replacement. All collected samples were analysed for retinol content by HPLC on the same day as the experiment. One set of experiments was performed with pure nanoparticle suspension versus free retinol and the other set of experiments with a serum containing 1.2% of retinol (free retinol equivalent of encapsulated retinol).

Skin penetration kinetics
Human skin explants were obtained from abdominoplasty of a 40-years old Caucasian woman and kept survival in physiological conditions. A topical cream containing encapsulated was applied on the surface of the skin and spread using a small spatula. The control explants did not receive any treatment. 3 topical formulas were applied on skin explants and contained either:
□ No retinol
□ Free retinol at 0.27 % (0.3 % is the maximum concentration authorised in leave-on/rinsed-off hand or face product in the EU),
□ Encapsulated retinol at 9 % (equivalent to 0.27 % free retinol)
Penetration kinetics were characterised 8 hours and 24 hours after application of the formulas.

3. Results

free retinol was shown to be highly unstable. Stability test demonstrated full degradation in less than 30 days for all samples, regardless of storage condition. Figure 1 presents the result.

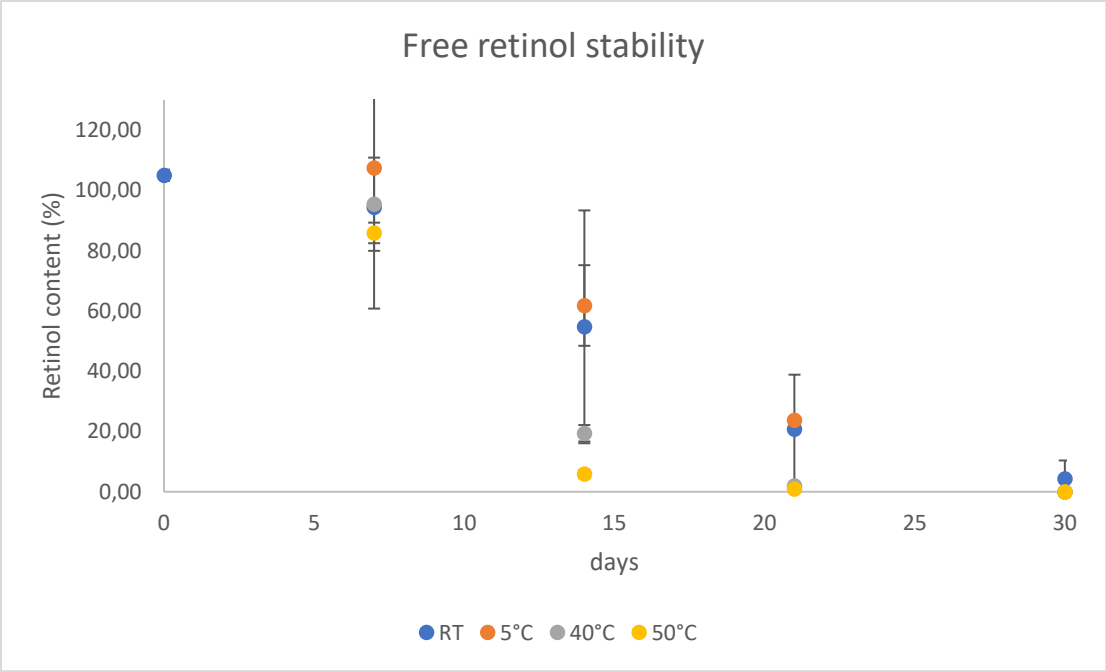

Figure 1. Stability profile of free retinol.

Immediately after preparation, the samples of encapsulated retinol were homogeneous, stable, without visible phase separation, light yellow in colour and without visible particles. The resulting final pH of the formulations was 8.2 ± 0.1.

The mean diameter D(50) was 0.28 ± 0.02 µm and the D(90) was 0.46 ± 0.01 µm. The span was 1.04 ± 0.09, indicating a narrow distribution. In the literature, a span below 2 is considered a low polydisperse distribution.

The average retinol content in the samples right after preparation was 3.58% ± 0.04 (m/v).

Figure 2 presents the data for the RT, 40°C and 50°C stability study.

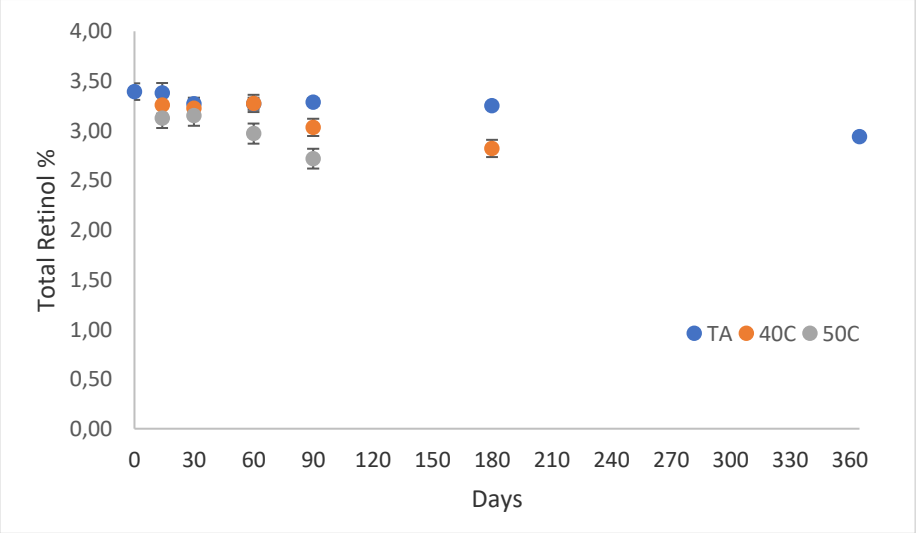

Figure 2. Stability of encapsulated retinol in terms of retinol dosage.

Particle size was also evaluated during stability. Particle size stability during storage conditions was adequate and Figure 3 shows the evolution of particle size over time.

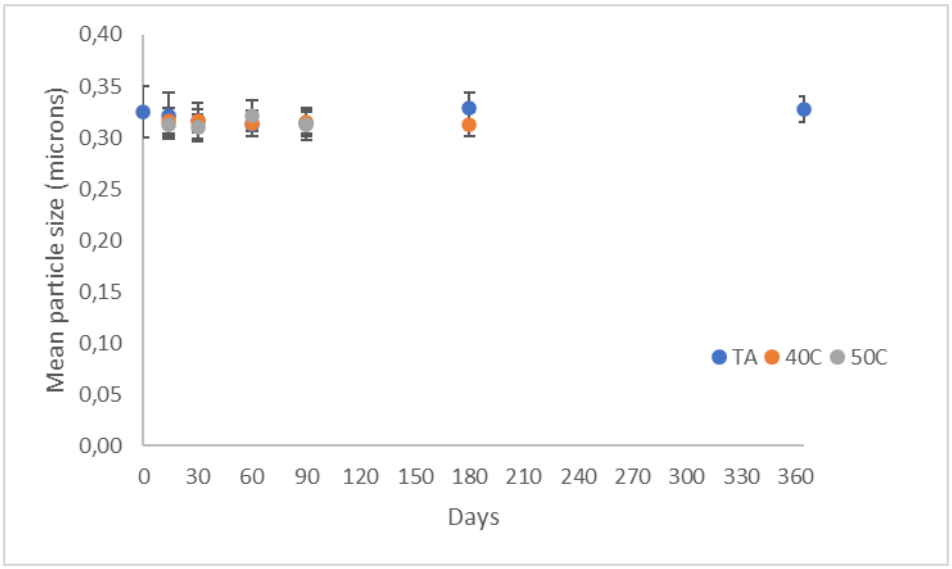

Figure 3. Stability of encapsulated retinol in terms of mean particle size.

Regarding encapsulation efficiency, peak area for retinol in the filtrate was zero, meaning no retinol remained or leaked out of the capsules into the aqueous phase. Hence, the calculated

encapsulation efficiency was 100 %. This experiment was repeated on free retinol solubilised in isopropanol (30:70 v/v) and confirmed all retinol was able to pass through the membrane, hence guaranteeing no retinol was adsorbed within the membrane/equipment thus masking the Cfree data.

To confirm the encapsulation efficiency, fluorescence images were obtained. The retinol molecule being naturally fluorescent, fluorescence microscopy was used as a visual method to prove retinol

encapsulation. Natural fluorescent molecules will appear black under normal visible light and coloured under fluorescent light, while non fluorescent molecules will appear black under both visible and fluorescent light. Figure 4 shows pictures of free retinol, encapsulated retinol, empty capsules (placebo formulation) and a blend of empty capsules and free retinol. The placebo formulation was prepared by substituting retinol with vegetable oil.

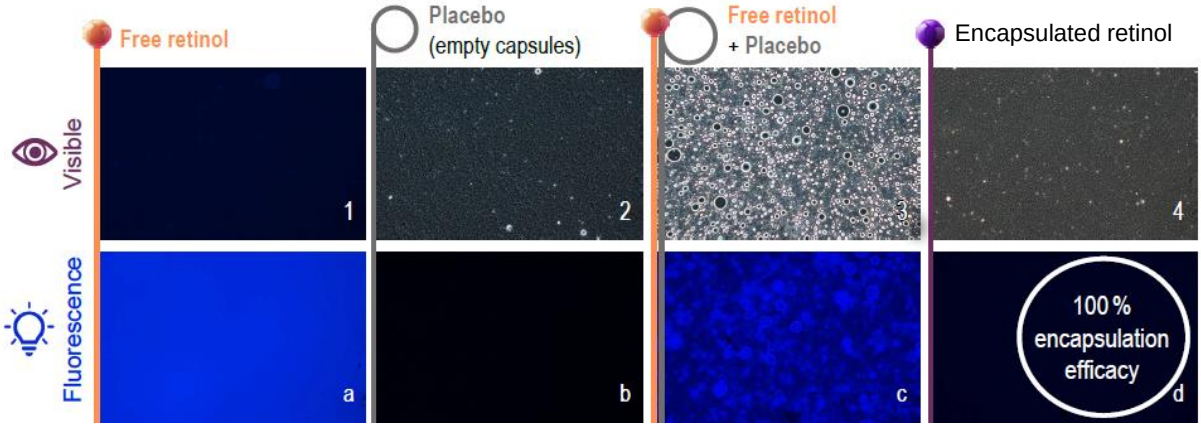

Figure 4. Light and fluorescent microscopy of free retinol, empty capsules, empty capsules added free retinol and encapsulated retinol.

The encapsulation in lipid particles allows the reduction in UV degradation of retinol (-20.36 %) compared to free retinol (-45.38 %).

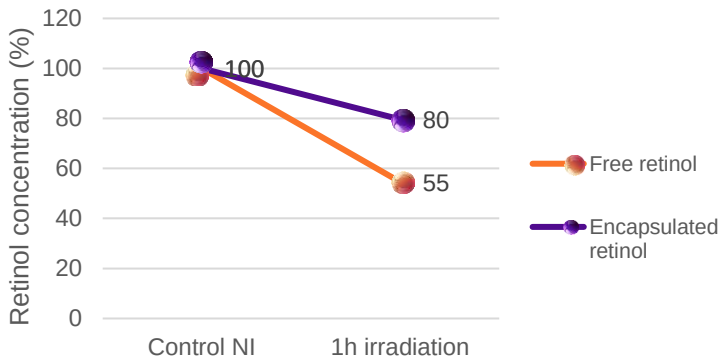

Figure 5. Retinol content after UV exposure.

Figure 6 shows the release profile obtained from free and encapsulated retinol. free retinol was released up to 24 h with increased concentration, however, the chromatograms showed degradation peaks after 10 h. Release of free retinol was faster than that of encapsulated retinol. After 24h, retinol was degraded almost to zero.

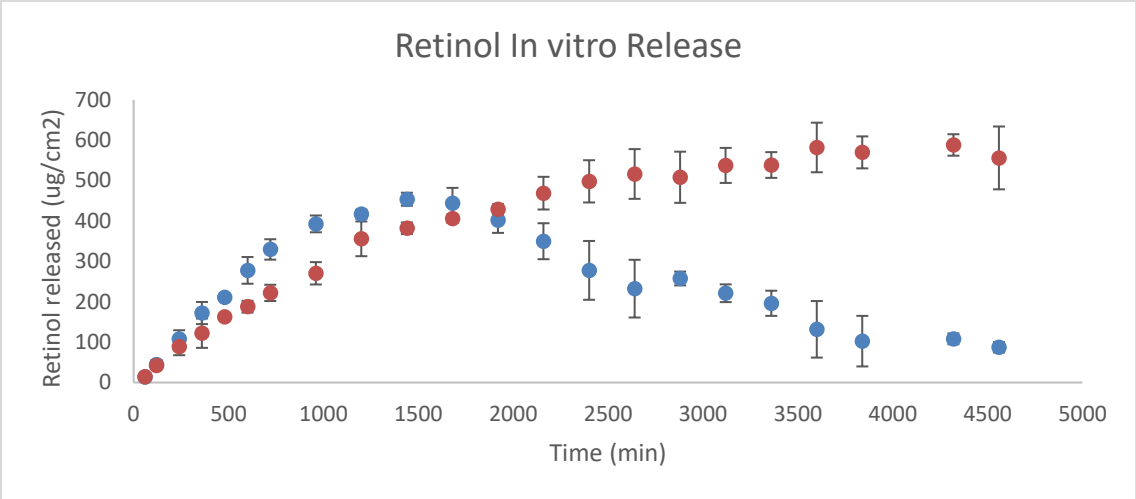

Figure 6. Release profile from free retinol (blue) and encapsulated retinol (red).

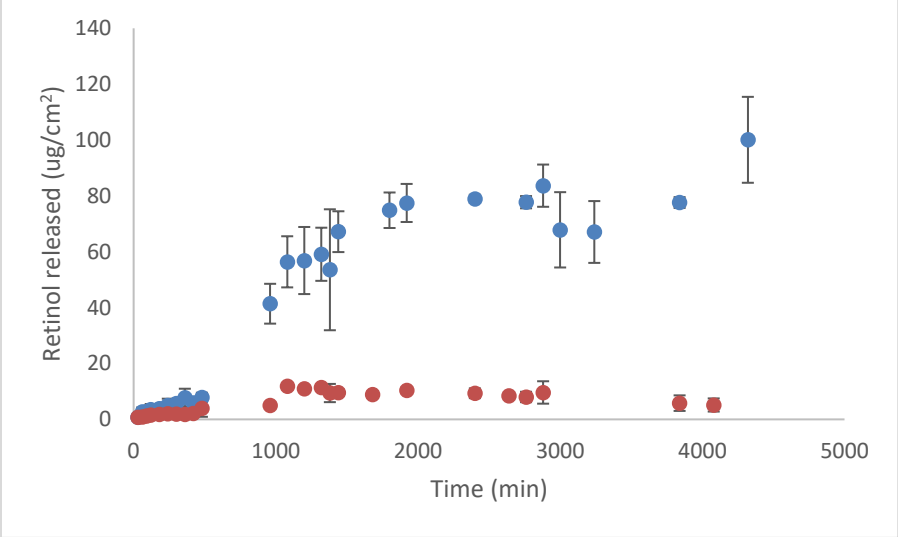

Figure 7. Release profile of retinol (red) and encapsulated retinol (blue) from a serum formulation.

Free retinol showed a small release from the serum with reduced total quantity released. Total retinol released from serum with the nanoparticles was 262 mg/cm^2 and from the serum with free retinol was 30 mg/cm^2.

Regarding skin penetration test, at 8 and 24 hours after application, no retinol-like signal was seen with the control. In skin explants treated with free retinol formula, a low signal was seen in the upper part of the stratum corneum after 8 hours. After 24 hours, retinol is concentrated in all the stratum corneum. Nothing was found into living epidermis.

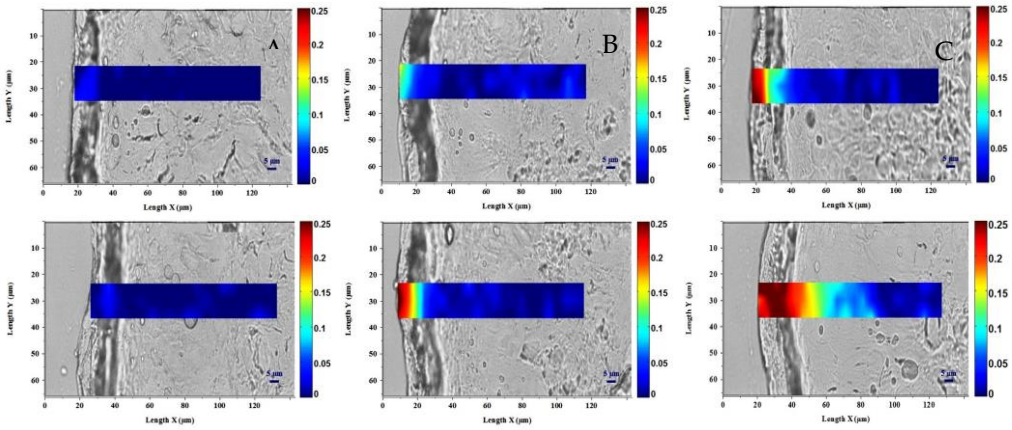

Figure 8. Skin permeation by Raman Spectroscopy A – placebo; B- free retinol; C- encapsulated retinol. Upper line results after 8h and bottom line after 24h.

4. Discussion

Retinol was successfully encapsulated and proved to be stable under all tested conditions and high levels after 12 months. The particle size was kept stable during all storage conditions.

Encapsulation efficiency was assessed by classical method of ultrafiltration and confirmed using an innovative approach of fluorescence microscopy. Images taken under standard visible light analysis are described as:

□ Images of free retinol (1) display a homogeneous dark colour, with no visible particles nor fluorescent blue.

□ Images of the placebo formulation as well as encapsulated retinol (2 and 4) display small homogeneous particles representing the capsules.

□ Images of the free retinol and placebo blend (3) show small homogeneous particles representing the capsules along with larger oil droplets representing free retinol dispersed in the water phase.

Images taken under standard fluorescent light analysis are described as:

□ Images of free retinol (a) display a characteristic homogeneous fluorescent blue colour.

□ Images of the placebo formulation (b) displays a homogeneous black colour and no fluorescence.

□ Images of the free retinol and placebo formulation blend (c) display large blue droplets demonstrating non encapsulated retinol fluorescence.

□ Images of encapsulated retinol (d) display an intense dark blue as the retinol fluorescence is greatly reduced by the shield from the capsule shell.

Based on the image analysis, it is possible to conclude that retinol is encapsulated in the capsules and that particle size is homogeneous. Indeed, no visible amount of free retinol is seen in the capsule microscopy images. Data from the two experiments confirm the encapsulation.

One benefit for encapsulation is protecting molecules from environmental conditions. In this experiment, UV light was used as stress agent. UV degradation was 2.2-fold slower with encapsulated retinol compared to the free active.

The nanoparticles released retinol in a slow and continuous process, reaching a retinol concentration greater than that of free retinol. In fact, peaks of retinol degradation were only visible after 24 hours and in a very small area compared to free retinol samples.
Encapsulated retinol was able to release higher concentration of retinol, 8.7 x more than the free version.
Analysis of skin explants treated with the product containing encapsulated retinol showed that retinol is concentrated in all the *stratum corneum* after 8 hours and 24 h after product application, retinol is located in both stratum corneum and living epidermis. Based on these results, encapsulation enhances skin penetration and tissue distribution of retinol making it more bioavailable compared to free retinol. These results correlate to the previous in vitro evaluations performed on PVDF synthetic membrane which showed that encapsulated retinol releases higher retinol concentration than free retinol.

5. Conclusion

Retinol is a widely used cosmetic active ingredient thanks to its multiple skin benefits and proven efficacy. However, this “hero ingredient” has many disadvantages being very unstable in environmental conditions (air, light, temperature) and causing skin irritation and photosensitization. Consequently, the instability of retinol makes it difficult to use and reduces its effectiveness in skin care products. The product developed in this study is a sustainably encapsulated form of retinol designed to meet the demand of the stable, easy to formulate, highly bioavailable and well tolerated retinol.
Under temperature variation, encapsulated retinol presents greater thermal stability. Unlike free retinol, which must be stored at 4°C under a N_2 atmosphere, encapsulated retinol can be stored at room temperature in its original closed container for up to 365 days.
In vitro skin release tests emphasized a slow release profile and greater stability even during the release study for encapsulated retinol. free retinol degrades during release. These results were confirmed ex vivo on human skin. Transcutaneous penetration studies have shown controlled release of retinol by forming a reservoir in the stratum corneum before penetrating living epidermis to a depth of 100 µm after 24 hours of application. Compared to free retinol, encapsulated retinol also showed greater skin penetration making retinol more bioavailable.
Based on these studies, we conclude that encapsulation improves the stability, bioavailability, and efficacy of retinol for advanced skin care.

6. References

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