Novel small sized Hyaluronic acid with enhanced therapeutic properties

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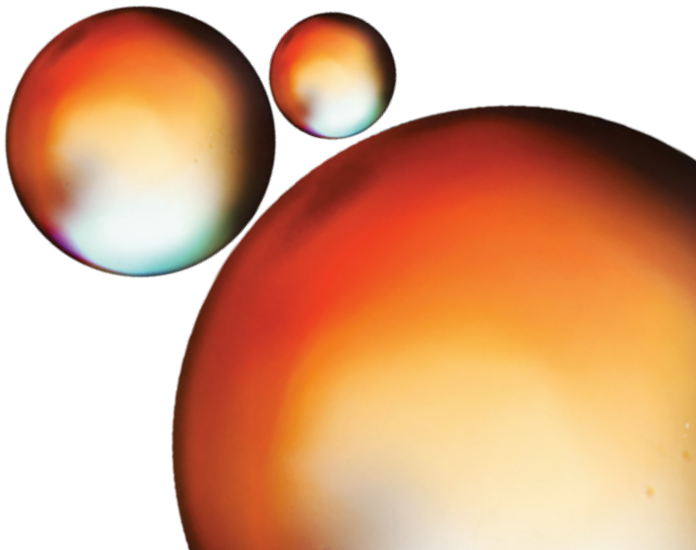
Abstract

Hyaluronic acid (HA) has several skincare applications. Primarily it is considered a moisturiser since it is a hydrophilic molecule, meaning that it attracts water. HA is also known as a free radical scavenger which makes it an excellent anti- aging agent. Topical HA in the form of gels, serums and so on is widely available but its usefulness is limited since it is a large polymer which cannot penetrate into the skin.

Several methods, including enzymatic and chemical means, have been applied to depolymerise HA into lower molecular mass fragments, but they are mostly time consuming, expensive and may result in residual concentrations of the reacting chemicals remaining in the therapeutic product. Lowering the size of a substance has an additional advantage because when the particle size decreases, its surface area increases, leading to a greater biological activity per given mass compared to larger particles.

Recently, micronized HA were prepared using a new technique. The particles sizes were evaluated using electron microscopy techniques and were found to be between 50 to 100nm in diameter. The antioxidant capability, as well as the hygroscopic properties (ability to attract water) of the micronized HA were found to be greater than their bulk counterparts. By all means the potential applications of this micronized HA in skin care products are huge.



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Introduction

Hyaluronic acid (HA) is a substance naturally present in the human body. In the skin, it fills the space between collagen and elastin fibers. Hyaluronic acid is a linear polysaccharide with the repeating disaccharide, β-1,4-D-glucuronic acid - β-1,3-N-acetyl-D-glucosamine. The size of HA may range from 100 kDa in serum to 8,000 kDa in the vitreous. Currently, hyaluronic acid has several skincare applications. Primarily it is considered a moisturizer since it is a hydrophilic molecule, meaning that it attracts water. HA is also known as a free radical scavenger. Production of free radicals such as superoxide, hydroxyl radicals, hydrogen peroxide (commonly designated as reactive oxygen species, ROS) is known to cause multiple skin damage.

Topical hyaluronic acid, in the form of gels, serums and so on is widely available but its usefulness is limited since most likely, HA will not penetrate the dermis sufficiently. In recent years, many efforts have been made to find ways to infiltrate materials into the human body (Sahoo, 2007), yet, reducing the size of HA remains a challenge.

Many methods have been applied to depolymerise HA into lower molecular mass fragments such as oxidative degradation (Hokputsa 2003), high temperature in an autoclave (Bothner 1998), and acid hydrolysis (Tokita 1995). Depolymerisation of HA was also achieved by enzymatic or other chemical means. Enzymatic methods for degrading hyaluronic acid are relatively inconvenient, and chemical methods suffer similar problems and, in addition, may result in residual concentrations of the reacting chemicals remaining in a therapeutic product.

Lowering the size of a substance has additional advantages. It is known that when the particle size decreases, the proportion of the surface area increases, which predicts a greater biological activity per given mass compared to larger particles (Ansari 2010). Consequently, we searched for new ways to reduce the size of polysacccharides characterized as hygroscopic (ability to attract and hold water molecules) and antioxidant natural products. It is also important to mention that hyaluronic acid accelerates wound healing by transporting growth factors and promoting angiogenesis. Lowering its molecular weight will obviously intensify this phenomenon (West 1985).

Recently we have developed an innovative technique to prepare Micronized particles of organic materials (NOPs). The NOPs were found to possess an increased biological activity as well as increased penetration into the tissue, the properties of micronized HA prepared using the new technique is discussed. Particles obtained were characterized by electron microscopy ESEM (Environmental Scanning Electron Microscope) and TEM (Transmission electron microscopy), Electron spin resonance (ESR) and by TGA (Thermal Gravimetric Analysis) analyses. The scavenging capabilities of hydroxyl radical, as well as the hygroscopy of the NOPs HA were shown to be higher than their bulk counterparts. By all means this is an important breakthrough of the potential applications of micronized HA in skin care products.

Materials and Methods

DETERMINATION OF HA SIZE

Scanning Electron microscopy (SEM)

SEM imaging was performed using the Environmental SEM (ESEM). The environmental scanning electron microscope allows the option of collecting electron micrographs of specimens that are "wet" by allowing a gaseous environment in the specimen chamber. Sample analysis was done using ESEM (Quanta FEG, FEI).

Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) is a microscopic technique where a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or detected by a sensor such as a CCD camera. TEM has been used for 2D imaging of the various HA samples. The size of the HA NPs was calculated using TEM (JEM-1400 JEOL).

DETERMINATION OF ANTIOXIDATIVE CAPABILITY OF HA

Electron spin resonance (ESR) spectroscopy

ESR (also known as EPR - Electron paramagnetic resonance) is a very sensitive technique for determining ROS like for example the presence of hydroxyl radicals.In the present study hydroxyl radicals were generated by performing a Fenton reaction.

$$Fe2+ + H2O2 \rightarrow Fe3+ + OH^* + OH-$$

Since hydroxyl radicals have a very short half-life (ns-ms), making them very difficult to detect directly, there is a need to add a spin trap which bounds the hydroxyl radicals, to give a long-lived free radical, called a spin adduct, that can be detected by the ESR technique (Buettner 1987).

We used 5,5 dimethyl-1-pyrroline-N-oxide (DMPO) to trap hydroxyl radicals to yield DMPO-OH that has a quartet signal in the EPR spectrum Figure 1 shows a typical ESR spectrum of DMPO-OH adduct which is characterized by its 1:2:2:1 quartet of lines and hyperfine splitting constant aN and aH=14.9. The area under the quartet signal is proportional to the amount of the hydroxyl radicals.

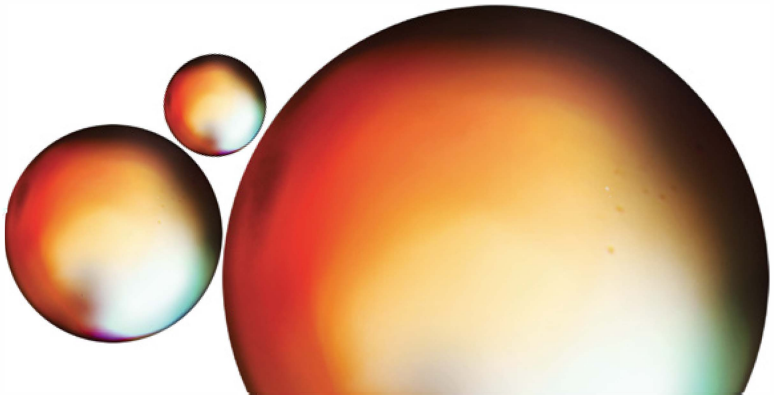


FIGURE 1: Typical DMPO-OH ESR spectrum.

When the Fenton reaction is performed in the presence of HA, there is a decrease in the quartet signal which indicates its antioxidative activity. The antioxidative properties of micronized hyaluronic acid relatively to that of intact HA was measured by the reduction of hydroxyl radicals generated in a Fenton reaction in the presence of Micronized or intact HA.The effect of hyaluronic acid on the formation of hydroxyl radicals was investigated by adding HA to a Fenton reaction system (H2O2+ FeCl2 + 4H2O +DMPO+ ddH2O/HA).

In order to detect hydroxyl radical coupled with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 0.02 M) (Sigma, St. Louis, MO), Fenton reagents with HA were drawn by a syringe into a gas permeable Teflon capillary (Zeus Industries, Raritan, NJ) and inserted into a narrow quartz tube that was kept open at both ends. The tube was then placed in the EPR cavity and the spectra were recorded after 5 minutes reaction time, on a Bruker EPR 100d X-band spectrometer. The EPR measurement conditions were as follows: Frequency, 9.74 GHz; microwave power, 20 mW; scan width, 65 G; resolution, 1024; receiver gain, 2 × 105; conversion time, 82 ms; time constant, 655 ms; sweep time, 84 s; scans, 2; modulation frequency, 100 kHz.

DETERMINATION OF HA HYGROSCOPY

The hygroscopic ability of hyaluronic acid was measured by dehydrating HA (using a lypophilyser) followed by incubation for 24h in an atmosphere of (1) 55% humidity, (2) 95%. The amount of the absorbed water by micronized HA relatively to intact HA was assessed using TGA analysis.,

Thermogravimetry/ thermal gravimetric analysis (TGA)

TGA indicates weight changes of samples. In the present study we used a Mettler Toledo TGA/STDA 851 instrument.

Results and Discussion

MICRONIZED HYALURONIC ACID SIZE

The main purpose for reducing the size of HA is to facilitate its delivery to human tissues and to enhance its penetration across the skin barrier. In addition decreasing HA size results in intensifying its characteristics like antioxidation capability and hygroscopy which are of great importance in medical cosmetics.

In the present research work we have used a new technique to produce a significant reduction in the HA size. The reduced sizes were evaluated using electron microscopy techniques. As shown in Figures 2 & 3, the intact irregular shape HA molecule of ~15X20 microns (circled, Figure 1a) went through a depolymerisation (degradation) procedure resulting in formation of micronized particles. The newly formed ONPs are supposed to penetrate the Stratum corneum barrier.

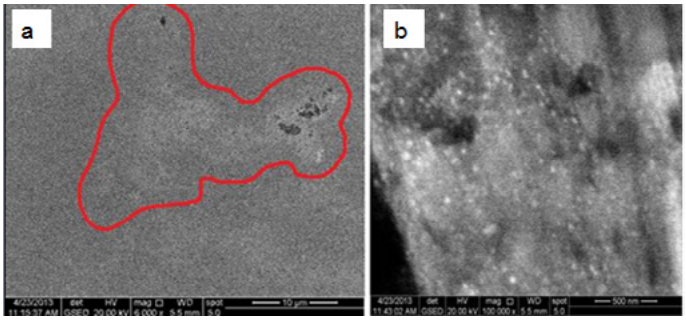


FIGURE 2: Scanning electron micrographs of hyaluronic acid micronized particles (b) and of intact HA molecule (a).

Very similar results were also observed by TEM of the samples (Figure 3). The images clearly show micronized particles formation in the modified HA. The HA micronized particles are of strict spherical shape. Size of the HA particles were micronized based on Bar-Ilan University Technology. A histogram showing the size distribution of the micronized HA is monitored in Figure 4. TEM image (Figure 3a) of the untreated sample clearly shows the irregular shape of the polymer, the different shades observed in the image are indicative of several layers of the polymer.

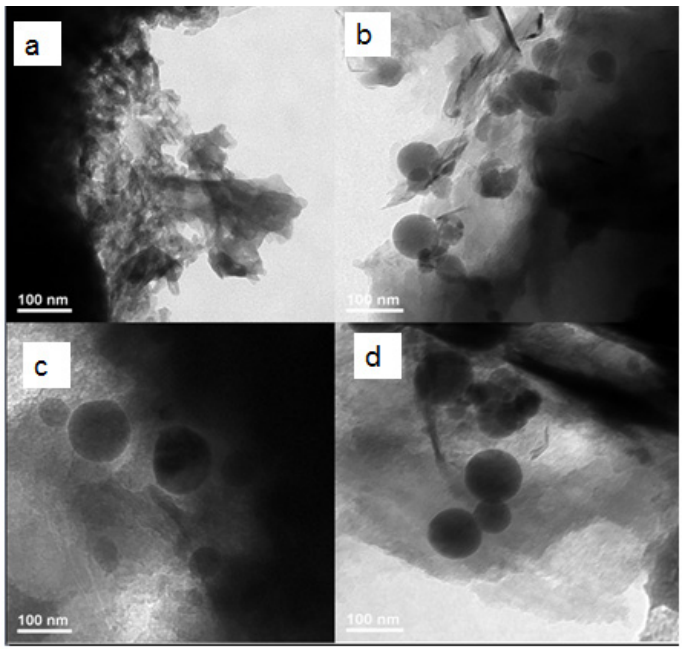


FIGURE 3: Transmission electron micrographs of (b-d) hyaluronic acid micronized particles and of (a) intact HA molecule (scale bar is 100nm).

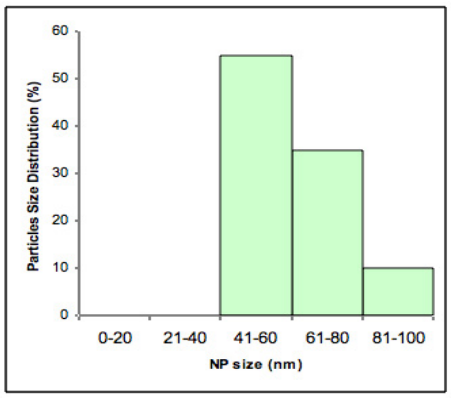


FIGURE 4: Particle size distribution of micronized - HA

Antioxidative activity of HA

Lot of attention has been paid to antioxidants that play an important role in preventing ROS-induced damage. HA is known for its antioxidative properties both in vitro and in vivo (Campo 2004, Balogh 2003). This makes HA a good anti-aging agent. By reducing the size of HA it is expected to increase its antioxidative capability. We therefore examined the antioxidative capability of micronized -HA prepared using our technique relatively to intact HA. As is demonstrated in Figure 5, micronized HA (Figure 5c) scavanges hydroxyl radicals better than untreated HA (Figure 5b).

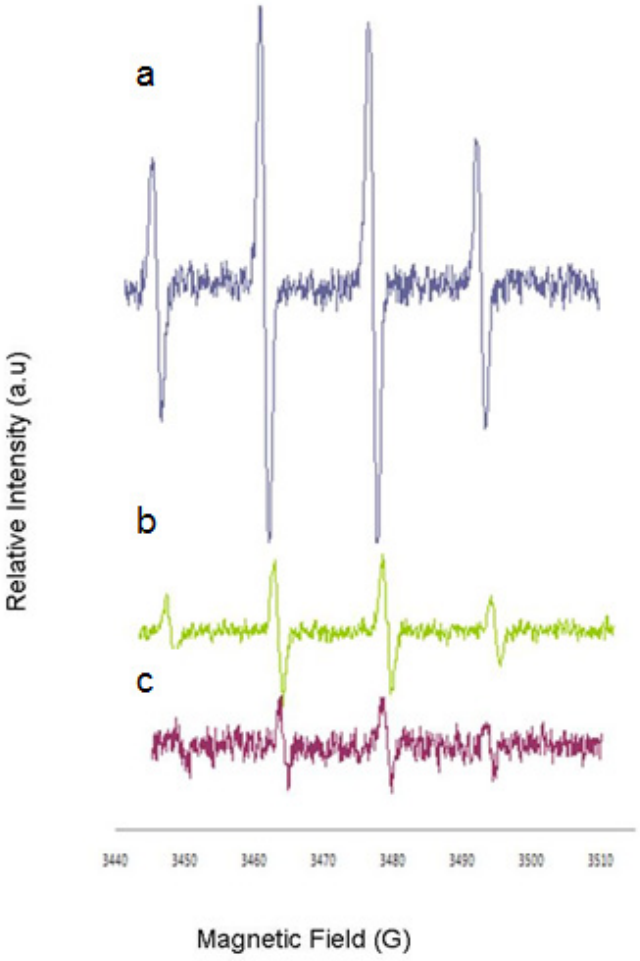


FIGURE 5: Hydroxyl radical formation via Fenton reaction in the presence of spin trap DMPO (a), intact HA (b) and micronized HA (c).

The ESR spectrum of DMPO-OH, (a quartet, which monitors the existence of hydroxyl radicals following a Fenton reaction) is shown in Figure 5a. Figures 5b,c depict the quartet indicative of hydroxyl radicals generated in a Fenton reaction in the presence of intact and micronized HA respectively. As can be seen from the decrease in the intensity of the quartet in Figure 5c, relatively to that in Figure 5b, the antioxidative capability of micronized HA is greater than that of intact HA. The intact and micronized HA antioxidative activity as a function of their concentration is depicted in Figure 6.

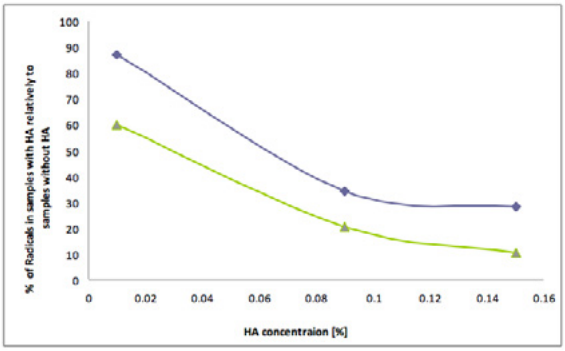


FIGURE 6: Hydroxyl radicals reduction by intact (blue) and micronized HA (green) as a function of the concentration.

Based on the integration area of the ESR quartet signals in Figure 5, the amounts of OH radicals generated by a Fenton reaction in the presence of micronized and intact HA relatively to those in the absence of HA were estimated. Figure 6 presents dose-dependent antioxidant activities of intact (blue line) and micronized HA (green line). It is clear that both intact and micronized HA show dose-dependent antioxidant activities with the micronized HA having a significantly stronger effect. For example 40% of hydroxyl radicals load was scavenged at 0.01% micronized HA solution, compared to only about 10 % scavenged by intact HA at the same concentration.

Preventing oxidative damage in living organisms by scavenging free radicals is of a great importance. The use of micronized HA that has been demonstrated to have a high radical scavenging effect relatively to intact HA would undoubtedly contribute to its use in skin treatments.

Hygroscopy

The biological functions of HA include tissue hydration. Its function in the human body is, amongst other things, to bind water and to lubricate movable parts of the body, such as joints and muscles. Its tissue-friendliness allows it to be used in skin-care products as an excellent moisturizer. The problem as discussed earlier is that HA is a huge polymer that cannot penetrate the skin. It is true that concentrated HA formulas can draw water from the air thus moisturizing the skin, but this should be avoided in dry climate. When air humidity is very low, HA may preferentially pull water from the skin rather than from the air, thus creating the opposite effect.

In the present research work, we examined the hygroscopic ability of micronized HA produced by using a new technique, relatively to intact HA.Absorption of water by intact and micronized HA was compared using the TGA system. Both intact and micronized HA were freeze dried using a Lyophilizer and then incubated in a 55% and 95% relative humidity environment for 24h. It was found that micronized HA absorbed 2.2 and 1.6 times more water at 55% and 95% humidity respectively, than the intact HA.

Summary

Hyaluronic acid is a legendary agent in the medical cosmetics area since it is an excellent moisturizer and also has a high antioxidative capability. The problem is its high molecular weight that prevents its use topically. It can be stated that externally applied hyaluronic acid in its conventional form i.e. as gels or creams is useless since it will not penetrate the stratum corneum barrier. To avoid injection, we prepared micronized particles of HA for easy skin penetration. Furthermore as has been shown in the present work reducing the size of the HA resulted in an increased antioxidative capability, as well as a higher hygroscopic property (ability to attract water) as compared with their bulk counterparts.Thus we expect the potential applications and benefits of this upgraded HA to be a big breakthrough in cosmetic dermatology.

CASE 1: Fine Lines & Wrinkles

Age: 50
Protocol: Use morning andevening after skin cleansing
Period: 6 1/2 weeks
Skin Type: type IV Regular/dry
Improvement: 47.5%

Before- Feature count 68

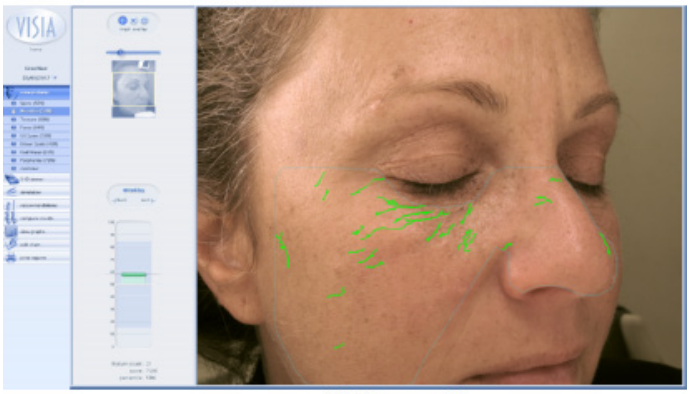
After- Feature count = 36

ited by Visia skin diagnostics system

CASE 2: Fine Lines & Wrinkles

Age: 50
Protocol: Use morning and evening after skin cleansing
Period: 6 1/2 weeks
Skin Type: type III Dry
Improvement: 47.6%

Before- Feature count = 21



After- Feature count = 11

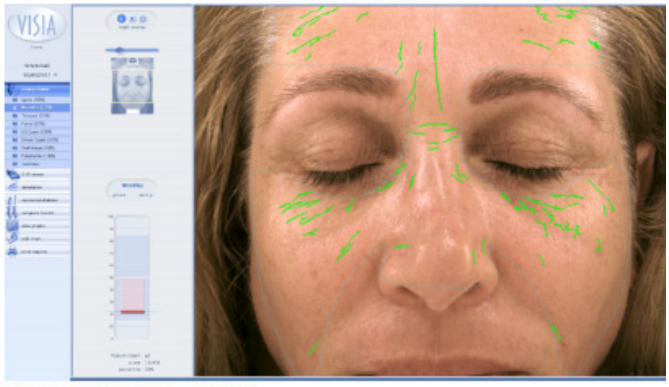
All Photos generated by Visia skin diagnostics system

CASE 3: Fine Lines & Wrinkles

Age: 46
Protocol: Use morning and evening after skin cleansing
Period: 6 1/2 weeks
Skin Type: type III
Improvement :46.5%

Before- Feature count 88

After- Feature count = 47



All Photos generated by Visia skin diagnostics system

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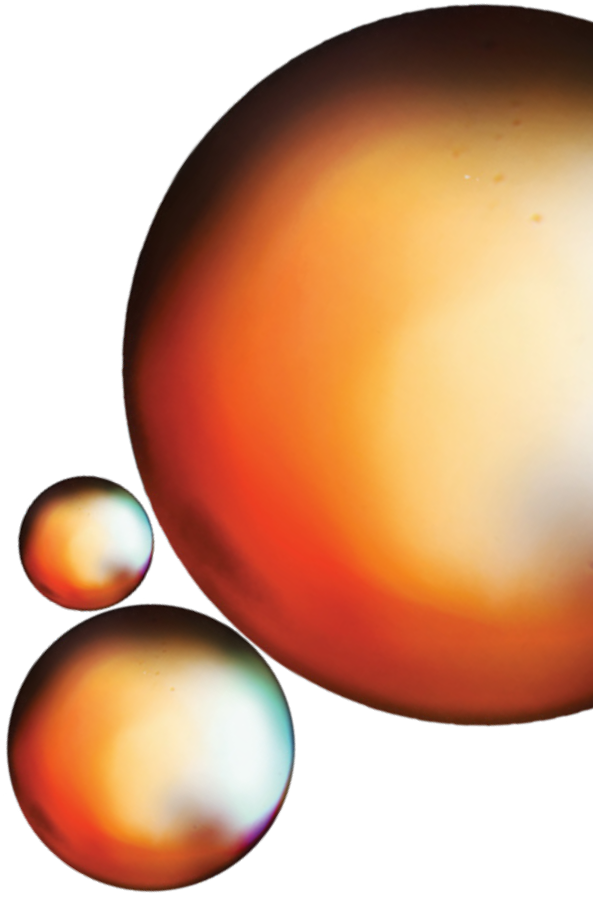


Prophecy Cream Proprietary Micronization Process:

First Evidence of Topical Hyaluronic Acid Skin Permeation

Introduction

During the last two decades, skincare companies have put tremendous efforts in research and development of new Hyaluronic Acid (HA) technologies, trying to find ways to effectively micronize HA molecules for topical applications. Driven by the growing demand for non-invasive HA technologies, many have tried to come up with a solution that will allow needle-free administration of functional HA molecules into the skin. However, in most cases these attempts were futile or only partially effective.

Today, after seven years of clinical research in the most advanced institutions of chemistry and nanotechnology, a ground-breaking, one-of-a-kind technology has been developed to successfully address the challenge. This technology, which was first implemented in the Prophecy Cream, has been proven to effectively increase the levels of HA within the inner layers of the skin.

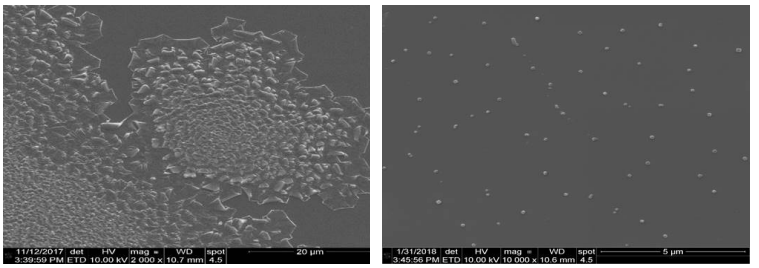
The Challenge

It is widely accepted that intact HA molecules, naturally weighted 2000-8000 KDa, are simply too large to penetrate the skin topically. To address this issue, researchers have developed depolymerisation techniques that allow production of much shorter and lighter HA polymers, which, in theory, have the potential to permeate the skin. Today, the most common raw material in HA moisturizers is a powder of dry HA molecules that were depolymerized into smaller molecules, to meet the demands of the client.

While dry HA depolymerisation is a relatively simple process, the more challenging aspect is keeping the HA molecules small and compact after they are formulized into the water-rich moisturizer medium. Once the dry micronized molecules are embedded in the cream, they immediately react with moisture and aggregate into bulky HA complexes which are, once again, too large to permeate the skin.

The Solution

The innovative HA micronization technology that is implemented in the Prophecy Cream allows for depolymerisation of large HA polymers into the desired smaller molecules after they are already formulized into the cream. In a sophisticated and unique process, the HA polymers in the Prophecy Cream are shortened, cross-linked, and packed into micronized structures which remain separated from each other. Upon contact with the skin, the micronized HA molecules can easily penetrate the stratum corneum and reach the deepest layers of the epidermis. The next microscopic images show a 50KDa HA formulation that was: a) not treated with Prophecy technology and b) was treated with Prophecy technology.



Left: 50KDa HA formulation that was not treated with Prophecy technology shows aggregation of HA molecules due to immediate reactions with moisture. These HA aggregations prevent skin penetration.

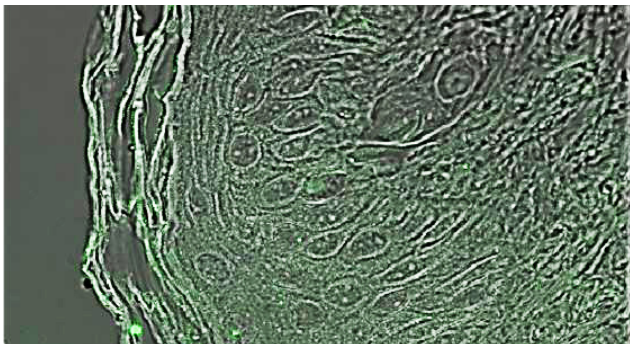
Right: 50KDa HA formulation that was treated with Prophecy technology shows separated HA molecules that can easily penetrate the skin barrier.

The images above demonstrate incompetence of low molecule weight HA to remain in a low-weight structure after formulation (left), whereas HA that was treated with the Prophecy technology remain micronized after formulation (right) and is ready to permeate the skin.

Skin Permeation Results

Evaluation of HA skin permeation was done ex-vivo by applying different HA formulations on porcine skin tissue for 5 hours, and marking of HA molecules with fluorescent markers to monitor their location in the skin.

The image below presents High Molecular Weigh HA cream that was not formulated with Prophecy micronization technology.



Above: Fluorescent microscopic image of High Molecular Weight HA shows negligible HA spots in the epidermis and stratum corneum.

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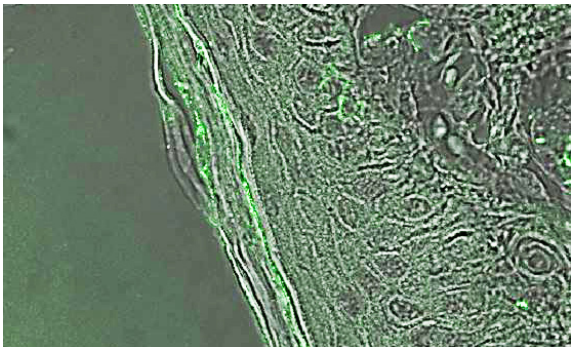
As expected, HMW HA molecules could not penetrate the skin barrier and were washed off the Petri dish.

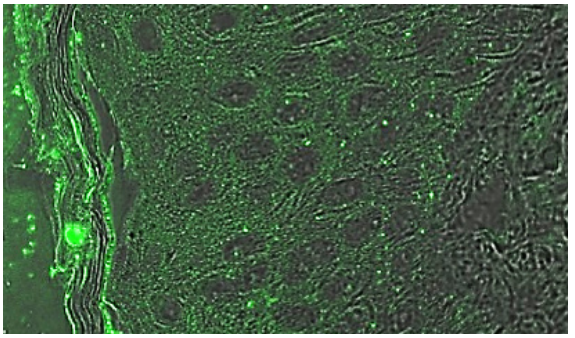
The next image presents Low Molecular Weight (50KDa) HA cream that was not formulated with Prophecy micronization technology.



Above: Fluorescent microscopic image of Low Molecular Weight HA shows HA stains in the stratum corneum.

As expected once again, the LMW HA formulation could not penetrate the stratum corneum and permeate to deeper layers of the skin. This result is attributed to aggregation of LMW HA that results in bulky complexes of HA.

Finally, the image below presents micronized HA formulation that was formulated with Prophecy technology.



Above: Fluorescent microscopic image of the Prophecy formulation shows significant HA stains in and out the stratum corneum and throughout the entire epidermis.

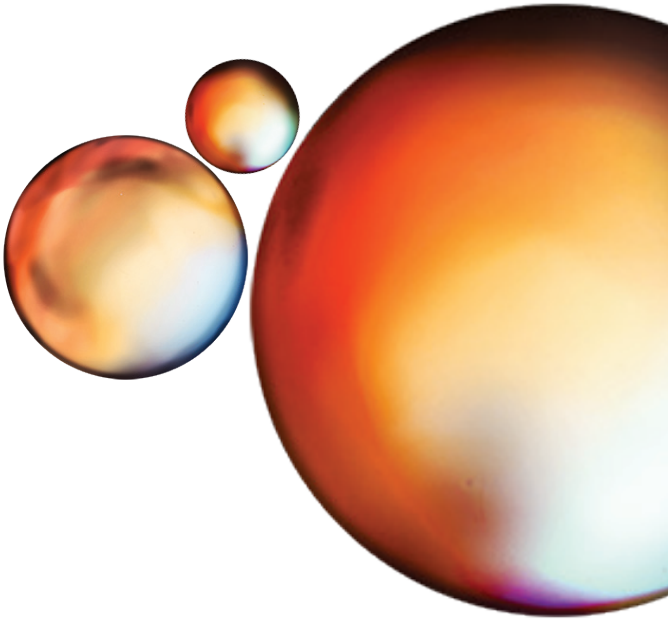
As opposed to formulations that were not treated with the Prophecy technology, here it can be clearly seen that HA molecules penetrate beyond the stratum corneum and enrich the deep epidermal layers. Bigger HA molecules that couldn't penetrate the skin remain on its surface and are not washed off.

Although keeping the HA molecules micronized after formulation and ensuring their skin penetration is a significant technological accomplishment by itself, it can't be considered an effective solution unless the micronized HA molecules maintain their native therapeutic properties.

A recent study (conducted by Prof. Rachel Lubart and her colleagues at the Chemistry Department, Faculty of Engineering and the Institute of Nanotechnology and Advanced Material of Bar-Ilan University, Israel) evaluated the therapeutic properties of micronized HA and compared them to those of intact HA molecules. The study found that micronized HA particles present higher ability to neutralize free radicals and have better hygroscopic capabilities (the ability of materials to retain moisture from their surrounding). These findings support the claim that the micronized HA particles of the Prophecy Cream maintain and even improve the therapeutic properties of high molecular weight HA molecules.

Conclusion

The Prophecy Cream is the first and only topical solution that allows needle-free administration of fully functional HA molecules into the deeper layers of the skin. The Prophecy Cream is based on an innovative and sophisticated technology that keeps HA molecules micronized after they are formulated into a cream, such that micronized structures of cross-linked HA permeate and enrich the epidermis to restore healthy, youthful skin. Prophecy's micronized HA molecules not only penetrate the skin, but they also maintain and even improve the therapeutic characteristics of intact HA molecules. It is not just another ingredient "mixology", but a scientifically proven technology that provides an effective multi-layered HA solution.



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