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In-vitro and in-vivo studies of excipient package compatibilities and functionality in transiting an undamaged stratum corneum

David Richard[1]

[1] RobinMcgrawRevelation, 9200 Sunset Blvd. Ste. 1212, West Hollywood, CA 90069, USA

Abstract: **The basic science technology of Dynamic Infusion Technology™ is demonstrated and the efficacy of a new transdermal delivery technology is evaluated in-vivo and in-vitro. Dynamic Infusion Technology has the ability to transport topically applied constituents to the intact epidermis without predicate damage to the stratum corneum. The product line includes a series of easily applied lotions and creams. The proprietary delivery system was developed from the company's previous research and product development in liposome and vesicle delivery of active ingredients. The transit of a large molecule, hyaluronic acid (HA), as well as additional active molecules such as argireline, beta glucan, tocopheryl acetate, ascorbyl palmitate, and niacinimide into the skin was demonstrated without the use of a treatment modality. The proprietary formulation of vesicles within the host serums, lotions, and creams allow the transit of HA into the skin, which helps with collagen remodeling and skin plumping.**

Keywords: **Transit, Delivery, Formulation, Polymers, Skin Barrier.**

I. INTRODUCTION

The stratum corneum is the skin's chief barrier that prevents the transit of various exogenous materials into the viable epidermis and dermis. The stratum corneum is predominantly composed of corneocytes (flattened cornified cells) and has a low water content of up to approximately 15 wt%. Therefore, the dominant route for the transit of molecules into the skin is transfollicular liposomal based formulations [1]. Furthermore, there is also a size exclusion criterion that applies to the transit of molecules into the skin; the stratum corneum with retained barrier function will not allow the transit of molecules or materials beyond a certain size to maintain its function [2]. Therefore, the entry of a relatively large molecule, such as hyaluronic acid (HA), has historically been found to be extremely difficult [3].

HA is a naturally occurring biodegradable polymer with a variety of applications in medicine including scaffolding for tissue engineering, dermatological fillers, and skin hydration enhancement. HA molecules can absorb up to 3,000 times their own weight in water. Therefore, HA has an important role in tissue regeneration and contributes to the regulation of wound healing, primarily through its hydrophilic properties and ability to be used as a lubricating and hydrating agent. In wound healing, HA serves two important functions: First, HA provides a temporary structure during the early stages of the wound healing [4]; this temporary structure diffuses nutritional supplies and helps rid the wound of waste products from cell metabolism. Second, HA is involved in keratinocyte proliferation and migration [5]. Eventually, the temporary structure is replaced by the protein molecules proteoglycans and collagen as the wound matures and the HA is degraded. As this process occurs, more protein molecules are produced. These proteins bind to HA to become proteoglycans and promote the healing process to build up tissue resilience [6].

Photoaging is one of the most common aging mechanisms known in skin conditions. Among various common conditions are solar elastosis, pigmentary dyschromia, lentigines, and rhytids, collectively culminating as chronological and environmental aging. To reduce and repair the changes associated with photoaging, nonsurgical options include chemical peels and treatment with chemicals that have minor irritant properties (e.g., topical retinoids, salicylic acid, and alpha-hydroxy acids). The process is based on the principle of wounding the stratum corneum—the skin's primary defense

against the transit of exogenous materials into the epidermis and dermis—to allow the penetration of constituents through the disrupted skin, which stimulates the desired response, typically restorative healing. All of these techniques require a wound healing response as a method to initiate the rejuvenation process [7]. For example, topical retinoids are an effective and common nonsurgical treatment for photoaged skin [8]. Topical retinoids work by decreasing the cellular rate atypia and by creating a more normalized, compact stratum corneum. Additionally, with the application of topical retinoids, keratinocyte differentiation is more organized and collagen synthesis is increased, resulting in smoother and softer skin texture [9, 10]. However, the application of retinoids is commonly associated with adverse events (AEs) when the product constituents compromise the stratum corneum. AEs include photosensitivity and a burning erythema, sometimes accompanied by edema, at the sites of application. Photoprotection, usually sunscreens, are often necessary to counteract the partial loss of sun protection from the retinoid-induced thinning of the stratum corneum [11].

Some traditional transdermal delivery mechanisms such as percutaneous injections (as in the case of dermal fillers), microneedling (as in mesotherapy), iontopheresis, transdermal patches, and liposomal formulations have been demonstrated to show efficacy of application. More recent advances have shown efficacy with the use of energy-based techniques such as fractionated laser, but these continue to be techniques that warrant a wound healing response. Additionally, all the aforementioned techniques are primed for enhancing the transit of either small or lipophilic molecules.

In addition to the delivery mechanisms, the type of molecule being transited into the skin is of critical importance. Traditional techniques, which are primed for enhanced transit of lipophilic molecules, do not necessarily help with the delivery of hydrophilic molecules. This is especially true when the target area is not the stratum corneum or epidermis, but rather the dermis, in which critical aging structures such as elastin and collagen are present. This is because the dermis is primarily composed of water and is more conducive to the rapid uptake of hydrophilic molecules.

A new approach for the transit of molecules in this application is the technology of delivery of HA into the skin to hydrate and stimulate the skin's formation of new collagen. Alternative techniques to injectable fillers are cosmetic infusion masques, which not only serve to provide cooling and hydration but also may contain active molecules such as HA. The challenge has been to deliver the molecules into the epidermis across an intact, undamaged stratum corneum.

In this study, we use a proprietary precursor formulation that also comprises penetration enhancers for HA and assess the transit of HA into human skin specimens. In addition to investigating the transit of a large molecule, we also investigated key active ingredients that are smaller in size but pertinent to improving skin efficacy and turnaround.

II. MATERIALS AND METHODS

A. In-vivo studies

1) Materials

Serums, lotions and creams that incorporate HA (50 kDa and 800 kDa), argireline, beta glucan, tocopheryl acetate, ascorbyl palmitate, and niacinimide, were fabricated by Robin McGraw Revelation (West Hollywood, CA). Tissue-Tek® O.C.T Compound was obtained from Sakura Finetek USA, Inc. HPLC grade water and acetonitrile were obtained from VWR International, Inc. All other chemicals used in this study, not specifically identified, were also obtained from VWR International, Inc. Human abdominal skin samples obtained from post abdominoplasty procedures were used in this study for the assessment of skin permeation. No human subjects were enrolled in this phase of the in-vitro proof of concept studies.

A precursor of HA in solution was prepared by using amphiphilic polymers to form an elastic vesicle structure in an aqueous solution because the water solubility of their hydrophilic moiety significantly differs from that of their hydrophobic moiety. In the aqueous solution, the vesicle has a unique multi-core-shell structure, wherein the hydrophobic moieties form an inner core and the hydrophilic moieties form an outer shell. The inner cores of such vesicle is filled with water-insoluble HA, thereafter which shows a significantly enhanced water solubility and an extended duration of any extended swelling effect. Furthermore, it is possible to control distribution in the skin depending on the size of the elastic vesicle and to deliver HA onto a target depending on the surface properties. Using this method of elastic vesicle construction, we are able to provide a method of delivering HA Stratum Corneum transit by using a vesicle that consists of a shell region comprising hyaluronic acid (HA) and a core region comprising a water-insoluble peptide with a terminal amine group, wherein the water-insoluble peptide is bound to the hyaluronic acid. This provides a delivery composition

comprising an elastic vesicle consisting of a shell region comprising hyaluronic acid (HA) and a core region comprising a water-insoluble peptide with a terminal amine group, wherein the water-insoluble peptide is bound to the hyaluronic acid inside the vesicle. The delivery composition, the method of producing the HA- loaded, hyaluronic acid-conjugate vesicle, and the method by using the vesicle is where the biocompatible, biodegradable hyaluronic acid-peptide conjugate vesicle is loaded with the water-insoluble HA (i.e., the active component), and the water insoluble peptide with a low bio-absorbability can be effectively dispersed in an aqueous solution such that its water solubility is greatly enhanced and the duration of the effect is extended.

The HA precursor and its host serum of fatty acid esters, polypeptides, polysaccharides, antioxidants, and polymers was combined to affect a serum that is hydrated into the delivery serum, lotion, or cream host during the fabrication process to create effectively HA-infused carrier serums, lotions, and creams. The HA used was a polyanionic polysaccharide, that is, a thiol-derivatized hyaluronic acid.

2) Skin preparation

1. Upon arrival of fresh tissue, the skin specimens were placed between gauze pads that soaked with 10X PBS (with 0.2% sodium azide).

2. The arrangement was then placed into a Ziploc bag and stored in an −80°C freezer.

3. 12 hours prior to the experiments, the frozen skin specimens were removed from the −80°C freezer.

4. On the dissection board, using a scalpel the specimens were dissected into desired size pieces.

5. Using surgical scissor, the hypodermis layer was removed but retained full thickness of the dermis and epidermis.

6. A thin layer of wet gauze (hydrated with 10X PBS with 0.2% sodium azide) was placed on a digital hot plate

7. The tissue samples were placed in the laboratory incubator (maintained at 32°C) elevated the temperature to produce a steady state temperature of approximately 32°C on the skin surface

8. The skin surface temperature was monitored using a remote IR thermometer.

3) Application of test material

1. The moisture on the skin surface was gently wiped off with dry gauze and then cleaned with a surfactant (0.5% DPPC).

2. The test material was placed on the skin surface with the stratum corneum upward.

3. The Franz cell receptor chamber was filled (8 mL capacity) with the PBS solution (pH 7.4) with 0.2% sodium azide (w/v).

4. Each specimen was then placed over the receptor chamber (stratum corneum facing upward) making sure to cover the active area on the receptor. A magnetic stir bar was already being placed in the receptor cell ahead of time.

5. The material under test was placed flush over the skin on the stratum corneum and positioned accurately.

6. Each receptor cell was then capped off with a donor cell on top of the skin sample and tightened with a clamp.

7. All cells (skin permeation systems) were then placed on top of a magnetic stir plate (rotation speed 550 rpm) in an incubator preset at 32°C.

8. The digital timer was set for a countdown as ascertained by the test and the objective of the study.

4) Tape stripping method

1. When the test duration time (1 hour in all tests) expired, the skin samples were removed from the laboratory incubator.

2. On the dissection board, the material under test was carefully removed. The surface of the skin was then cleaned with a damp gauze followed by 99% IPA damp gauze.

3. Each skin specimen was visually inspected to determine if any residue remained.

4 Using tweezers, scotch tape was applied on the specimen with the sticky side on the stratum corneum and rapidly removed.

5. An untreated (no test material) control sample was also processed and analyzed under identical conditions as the test samples.

6. A total of 11 such tape applications were applied, the first of which was discarded owing to potential contamination of the skin surface.

7. The remaining tape samples were placed in 1.5 mL microcentrifuge tubes and subsequently extracted by vortexing at high speed for 1 minute followed by centrifugation at 12,000 rpm for 10 minutes at 40°C.

8. The supernatant solution was then drawn out of each tube/container, filtered and analyzed with an HPLC system to determine the amount of active ingredient retained in the skin specimen

9. Averages of all samples tested were taken and recorded.

5) Skin permeation method

1. A 500 µm thick strip of skin was heated to 32°C and then cut into 2 × 2 cm pieces.

2. Each piece was visually inspected for any defects, and any samples that appeared to be compromised were discarded.

3. The receptor chamber was filled (8 mL capacity) with the PBS solution (pH 7.4) with 0.2% sodium azide (w/v).

4. Each skin specimen was placed over the receptor chamber (epidermis facing upward) making sure to cover the active area on the receptor. A magnetic stir bar was already placed in the receptor cell ahead of time.

5. Each receptor cell was capped off with the material under test followed with a donor cell on top of the skin sample and tightened with a clamp.

6. All cells (skin permeation systems) were placed on top of a magnetic stir plate (rotation speed 550 rpm) in an incubator preset at 32°C.

7. Aliquots (1 mL) were extracted and fresh solution was correspondingly replaced from each diffusion cell at intervals of 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, and 24 hours.

8. The aliquots were filtered and analyzed using reversed phase HPLC.

9. After the 24-hour permeation test was complete, the skin graft under test was washed with 5% soap solution.

10. Then a biopsy punch (6 mm) was excised from the area in direct contact with the material under test and weighed (the weight of each sample was used to determine the approximate thickness of the sample, both of which were used for normalizations to ideal weight given an ideal thickness of 500 µm.

11. This biopsy punch was diced using a scalpel and then homogenized for approximately 60 seconds in 10 seconds pulses followed by centrifugation (4°C) at 10,000 rpm for 10 minutes.

12. The supernatant solution was then be drawn out of each tube/container, filtered and analyzed using HPLC for the amount of actives retained in the skin specimen under test.

13. Total uptake was calculated as the sum of the normalized cumulative permeation and the normalized retention in each sample.

14. Averages of all samples tested were taken and recorded.

6) Solution Sampling and HPLC parameters

1. Volume Extracted (mL), V_e: volume of sample solution extracted from each diffusion cell at each time interval

2. Diffusion Cell Volume (mL), V_o: original volume of solution within each diffusion cell

3. Replacement Volume (mL) V_r: volume returned to diffusion cell after each extraction, and has to be the same as Volume Extracted ($V_r = V_e$), to make up a total volume which must be equal to the original Diffusion Cell Volume

4. Injection Volume (mL), V_i: volume of sample solution injected for HPLC analysis

5. Dilution Factor, D: Magnitude of sample solution diluted before injection for HPLC analysis.

7) Calibration Curve

1. For each Molecule-Under-Test, a calibration curve was created with the HPLC system prior to the experiments. A few samples of the active under test were prepared at known concentration (mg/mL) which was analyzed with the HPLC system for chromatographic absorbance (mAU). The absorbance peak for the molecule was identified by its retention time in the separation column compartment.

2. A graph of a simple linear algebraic equation $Y = mX$ was plotted, in which Y = Absorbance (mAU) and X = Mass (µg). The slope, m was noted and used later to calculate the actual total mass of molecule permeated, $X_{t,n}$, through the skin samples during the experiments, given the absorbance measured by the HPLC system, in which t = sampling time interval and n = index number of the skin permeation system.

8) Analysis Template

At each time interval, V_e= 1 mL sample solution was extracted from each permeation cell, and V_r = 1 mL was returned to each permeation cell. An injection volume, V_i, for HPLC analysis from each sample solution was determined, along with an optional dilution factor, D. During each analysis, the HPLC system returned an absorbance value, Y. The actual volume or mass of the Molecule-Under-Test was calculated using the calibration curve. The volume or mass of sample solutions, $X_{1,n}$ taken on the first time interval was calculated using:

$$X_{1,n} = \frac{Y - C}{m} \cdot \frac{V_o D}{V_i}$$

The mass of sample solutions, $X_{t>1,n}$, taken on subsequent intervals was calculated using:

$$X_{t>1,n} = \frac{Y - C}{m} \cdot \frac{V_o D}{V_i} + \frac{X_{(t>1)-1,n} V_e}{V_o}$$

The permeation of the molecule under test per cm2 was calculated by knowing the effective area of the treated skin specimen in direct contact with the topical solution in the donor cell. The area of skin in contact with the topical solution was equivalent to the area of the opening slit on the donor cell.

Therefore, permeation $P_{t,n} = \frac{X_{t,n}}{A}$ mg/cm^2

in which A = area of skin in contact with molecule under test (1 cm diameter). The average of all the samples at each time point was taken and plotted with permeation of the molecule under test as a function of time. Based on the measurements and calculations, the samples evaluated at 24 hours provided a cumulative amount permeated past the skin graft under test. Because the graft thickness was known to vary, the permeation amount was normalized to a 500 µm skin graft thickness by using:

$$X_{24hours,n} = \left(\frac{Y - C}{m} \cdot \frac{V_o D}{V_i} + \frac{X_{(t>1)-1,n} V_e}{V_o}\right) \cdot \left(\frac{t_m}{t_i}\right)$$

in which t_m was the measured graft thickness and t_i was the ideal graft thickness, which was 500 µm. Averages of the samples were taken to determine the mean and standard deviation for permeation. The permeation ratio and ratio standard deviation could then be calculated. At the end of the permeation testing period (24 hours) a 6 mm biopsy punch was taken from the center of the skin specimen that was in contact with the topical solution under test. The biopsy was weighed in grams. Once the biopsy was processed and the resultant aliquot absorbance value was obtained, the mass of molecule retained was calculated using:

$$Z_{t,n} = \frac{Y - C}{m} \cdot \left\{\frac{V_c D}{V_i}\right\}$$

in which V_c = volume of supernatant in the centrifuge tube and t = 24 hours. This quantity was normalized for the area of the skin in contact with the molecule under test and for differential mass (weight of sample) compared with the ideal mass for a 500 µm graft thickness. This was calculated by

$$R_{t,n} = \left(\frac{Y - C}{m} \cdot \left\{\frac{V_c D}{V_i}\right\}\right) \cdot \left(\frac{A_N}{A_B}\right) \cdot \left(\frac{M_i}{M_n}\right)$$

in which A_N = normalized area of skin in contact with the molecule under test A_B = biopsy area , M_i = ideal mass for 500 µm graft thickness, M_m = mass measured on each biopsy . Averages of the samples were taken to determine the mean and standard deviation for retention. The retention ratio and ratio standard deviation were also calculated. Normalized uptake of the molecule under test was calculated by using:

$$U_{t,n} = X_{24hours,n} + R_{24hours,n}$$

for the mass of the molecule uptaken by the skin specimen in contact with the test solution. The uptake in mass per unit area was further calculated by:

$$U_{t,n} = \frac{\left(X_{24\ hours,n} + R_{24\ hours,n}\right)}{A}$$

in which A = the area of skin in contact with the molecule under testing. Averages of the samples were taken to determine the mean and standard deviation for uptake enhancement. The uptake ratio and ratio standard deviation were then calculated.

9) Histological processing

Biopsy specimens were taken from 1 hour exposure tests and embedded in Optimal Cutting Fluid Temperature fluid for frozen sectioning. Samples were frozen sectioned at a thickness of 10 µm and collected on charged microscope slides. Images were taken using a light microscope with a preinstalled digital camera (Leica, Inc.) using light and cross polarized filtering. In addition to the two molecular weights of HA tested (50 and 800 kDa), tests were also performed for individual constituents delivered through the following serums:

Serum 1: 5% Argireline (acetyl hexapeptide-8)

Serum 2: 2% Beta Glucan

Serum 3: 3% Tocopheryl Acetate

Serum 4: 3% Ascorbyl Palmitate

Serum 5: 5% Niacinimide

B. In-vivo studies

1) Clinical trials

Two products, a hydrating serum and a hydrating cream, were evaluated in 140 patients at 4 U.S. centers for 4 IRB approved treatment protocols. For the first protocol, the safety and efficacy of the serum and cream as an adjunctive agent in ablative or fractional ablative laser resurfacing. The other 3 protocols examined the deep hydrating and Crow's Feet cream as an adjunctive agent for cosmetic facial procedures, the deep hydrating serum as an adjunctive agent in microdermabrasion. Study objectives, methods, and results for each of the protocols are reported below.

Post-Treatment Protocol

The primary study objective of this protocol was to determine the effectiveness of the post-treatment serum, as rated by the physician investigator, in reducing healing time, redness, edema, and dried exudate when applied after an ablative or fractional ablative laser resurfacing procedure. The study's secondary objective was to measure patient satisfaction with the primary objectives, as well as satisfaction with the deep hydrating serum, which was applied at home over the next 3 days.

Evaluations were performed on treatment day (Day 0) and Days 1, 7, and 30. Patients kept a log to determine their satisfaction with the reduction of healing and their opinion of redness and edema, as described in the primary objective. Patient assessments, which included scaled surveys and write-in comment portions, were conducted on the follow-up evaluation days (Days 1, 7, and 30). On the treatment day, the investigator took high-resolution photographs of the subject's face before treatment using the VISIATM complexion analysis system or equally high- resolution photography. Photographs included a full frontal view, a 45° angle view, and one side view of each side of the face. After performing an ablative or fractional ablative laser resurfacing procedure the investigator took high-resolution photographs of the face and treated areas as was done before the procedure, and recorded assessments of the area of the wound, degree of redness, edema, and dried exudate.

The investigator then applied the post-treatment serum to the face and instructed the patient to use the serum 3 times in 24 hours. The patient was given sufficient serum and instructed to apply 3 times per day for 3 days. On follow-up evaluations (Days 1, 7, and 30), the provider took high-resolution photographs and recorded area of healing, redness, edema, and dried exudate. Patient-reported satisfaction was also recorded on these days using a patient evaluation form.

2) Deep hydrating protocols

The protocols using the deep hydrating cream had two study objectives: The primary objective was to determine the effectiveness of the deep hydrating cream in each protocol in reducing the appearance of fine lines and wrinkles as assessed by the investigator. In the microdermabrasion protocol, there was an additional objective of reducing post-treatment redness. The secondary objective was to measure patient satisfaction with each of the primary objectives.

Evaluations were performed on treatment day (Day 0), Day 3, and Day 7. Patients kept a log to determine their satisfaction with the appearance of fine lines and wrinkles. Patient assessments, which included scaled surveys and write-in comment portions, were conducted on the follow-up evaluation days (Days 3 and 7). On the treatment day, the investigator took high-resolution photographs of the subject's face before treatment using the VISIATM complexion analysis system or equally high-resolution photography. Photographs included a full frontal view, a 45° angle view, and one side view of each side of the face. The investigator recorded the baseline assessment of the appearance of fine lines and wrinkles. After performing the aesthetic procedure, the investigator recorded a post- treatment assessment using the same parameters as the pretreatment assessment. Next, the investigator applied the Deep Hydrating cream once every 4 hours (every 3 hours for microdermabrasion). For the lip and crow's feet protocol, patients were given the deep hydrating cream and instructed to apply it once daily at night for the next 2 nights. For microdermabrasion, patients were given the cream and instructed to apply it every 3 hours over the next 2 days. Inclusion and exclusion criteria are summarized in Table I and II.

Table I: Inclusion and Exclusion Requirements: Post-Treatment Serum Protocol

Inclusion Criteria	**Subjects who met all of the following criteria could be included in the study**
1	Females age 18 years or older
2	Able and willing to provide written acknowledgment of participation
3	Able to apply cream reliably, as recommended by the provider, either by self or with available assistance
4	Able and willing to maintain patient log for reporting results
Exclusion Criteria	Subjects who met any of the following criteria were excluded from the study
1	Have received surgical or nonsurgical cosmetic procedures (including facials) at any time during the 4 weeks prior to initiation of the study
2	Are scheduled to receive surgical or nonsurgical cosmetic procedures at any time over the duration of study (30 days)
3	Are pregnant, lactating, or planning to become pregnant
4	Have an open or healing lesion, rash, or other irritation on the face
5	Have or have had a skin disorder that may confound measurement of effectiveness variables or render subject susceptible complications from ablative or abrasive resurfacing procedures (e.g., skin cancer, scleroderma, dermatitis)
6	Have severe active facial acne
7	Are unable or unwilling to avoid excessive sun exposure or the application of topical products that contain glycolic acid, alpha hydroxyl acids, or retinoids; over the course of study, must be willing to apply sunscreen daily

Table II: Inclusion and Exclusion Requirements: Deep Hydrating Cream

Inclusion Criteria	**Subjects who met all of the following criteria could be included in the study**
1	Females age 18 years or older
2	Able and willing to provide written acknowledgment of participation
3	Able to apply cream reliably, as recommended by the provider, either by self or with available assistance
4	Able and willing to maintain patient log for reporting results
Exclusion Criteria	Subjects who met any of the following criteria were excluded from the study
1	Have received surgical or nonsurgical cosmetic procedures (including facials) at any time during the 4 weeks prior to initiation of the study

2	Are scheduled to receive surgical or nonsurgical cosmetic procedures at any time over the duration of study (7 days)
3	Are pregnant, lactating, or planning to become pregnant
4	Have an open or healing lesion, rash, or other irritation on the face
5	Have or have had a skin disorder that may confound measurement of effectiveness variables (e.g., skin cancer, scleroderma, dermatitis)
6	Have severe active facial acne
7	Are unable or unwilling to avoid excessive sun exposure or the application of topical products that contain glycolic acid, alpha hydroxyl acids, or retinoids; over the course of study, must be willing to apply sunscreen daily

III. MATERIALS AND METHODS

A. In-vitro studies

Most of the studies were performed with 800 kDa HA. This was done to have uniformity in gauging the changes in uptake through penetration enhancer and excipient modifications. Some defining studies were performed to discern the difference in performance in the uptake of the 50 kDa and 800 kDa HA. Furthermore, a distinct transit evaluation of a combined content serum containing both 50 kDa and 800 kDa HA was performed. Furthermore, histological testing was performed on samples exposed to additional individual active ingredients, presented within the embodiment of the ensuing results. Quantitative measurements were only performed using HA and not assessed for the other active ingredients.

1) Tape stripping results

A common tissue donor was used with three independent site applications of the test material hosts were tested on different areas of the tissue specimens through an incubation period of 1 h. Both, the 50 kDa and the 800 kDa creams showed transit into the skin specimen at this incubation point (Figure 1). Also noted from the results was that the 50 kDa samples showed greater transit into the skin when compared with the 800 kDa samples. The cumulative transit into the skin progressively increased as a function of depth into the skin (Figure 2). This plot is simply an additive representation of the data shown in Figure 1.

Also tested within the paradigm were cream samples that were loaded with both isotopes of HA, 50 kDa and 800 kDa, all within the same samples. The test protocols followed were identical to those in which samples contained only one form of HA. The test results for individual transit and cumulative transit are shown in Figures 3 and 4, respectively. Despite the visible quantifiable transit of HA into the skin, the two isotopes were indistinguishable traces from one another.

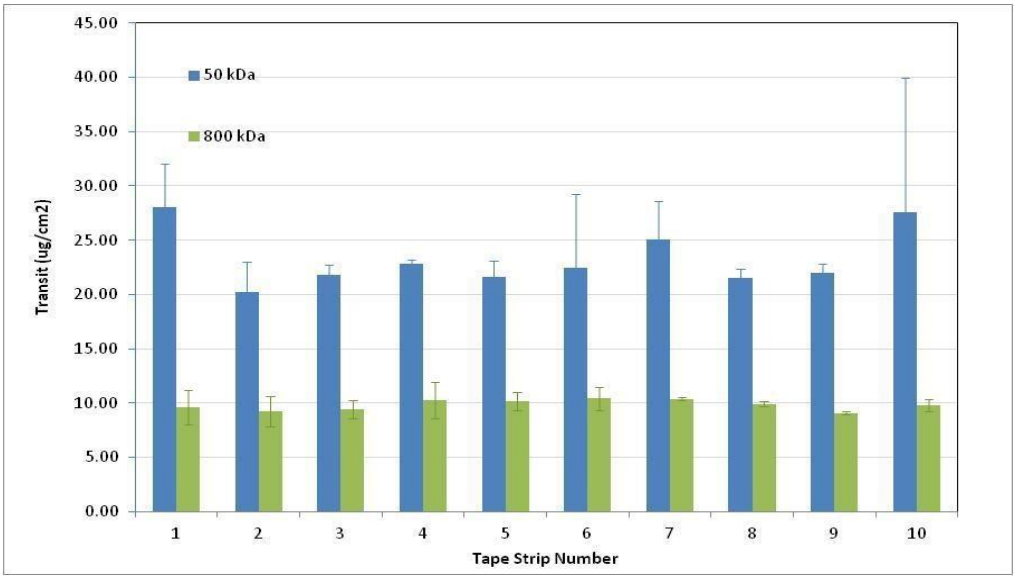


Figure 1: A plot showing the transit of the HA into the skin via tape strips collected from the surface of the skin specimen in contact with the HA cream for an incubation duration of 1 h. Note the greater transit in the 50 kDa samples, while still significantly visible transit with the 800 kDa samples.

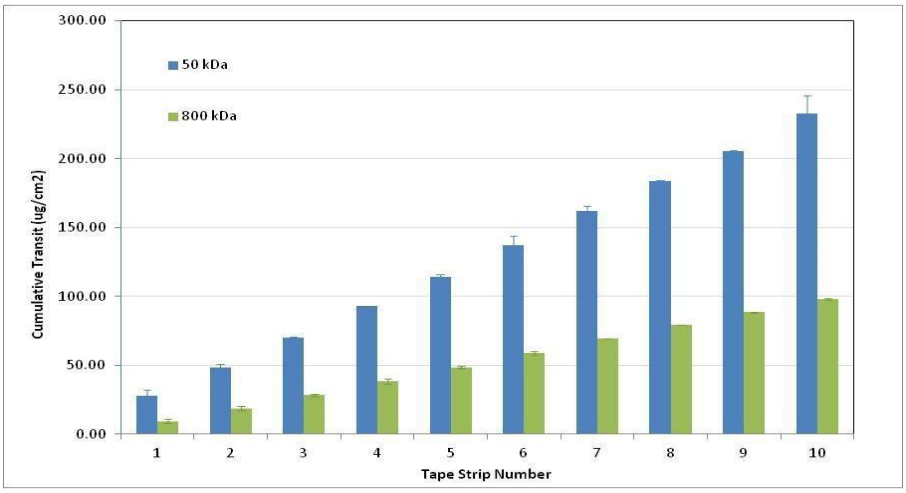


Figure 2: A plot showing the cumulative (additive) transit of the HA into the skin through tape strips collected from the surface of the skin specimen in contact with the HA cream for an incubation duration of 1 h. Note the greater transit in the 50 kDa samples and a significantly visible transit with the 800 kDa samples. This plot is simply an additive representation of the data shown in Figure 1.

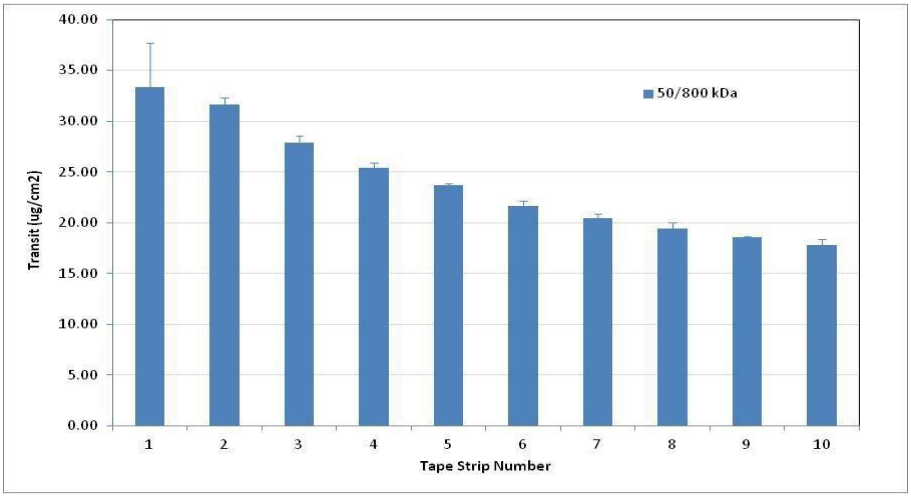


Figure 3: A plot showing the transit of the combined 50kDa/800 kDa HA into the skin through tape strips collected from the surface of the skin specimen in contact with the HA cream for an incubation duration of 1 h.

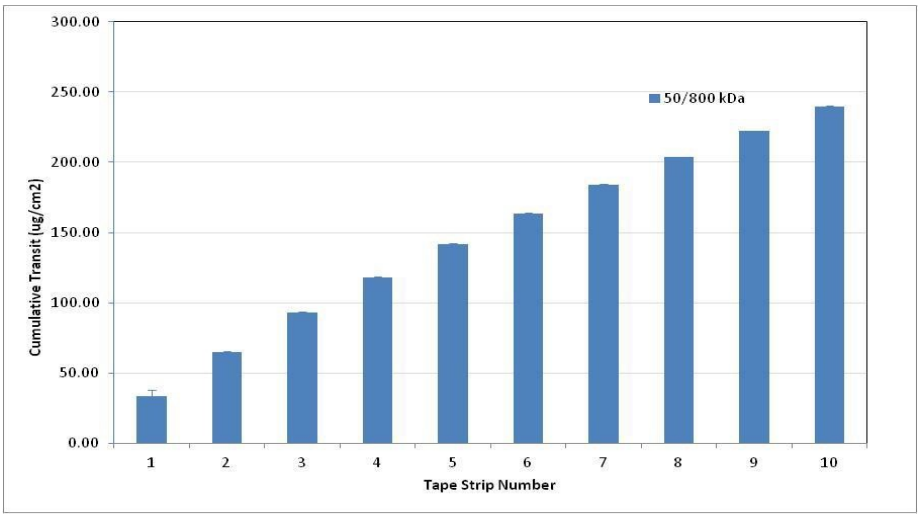
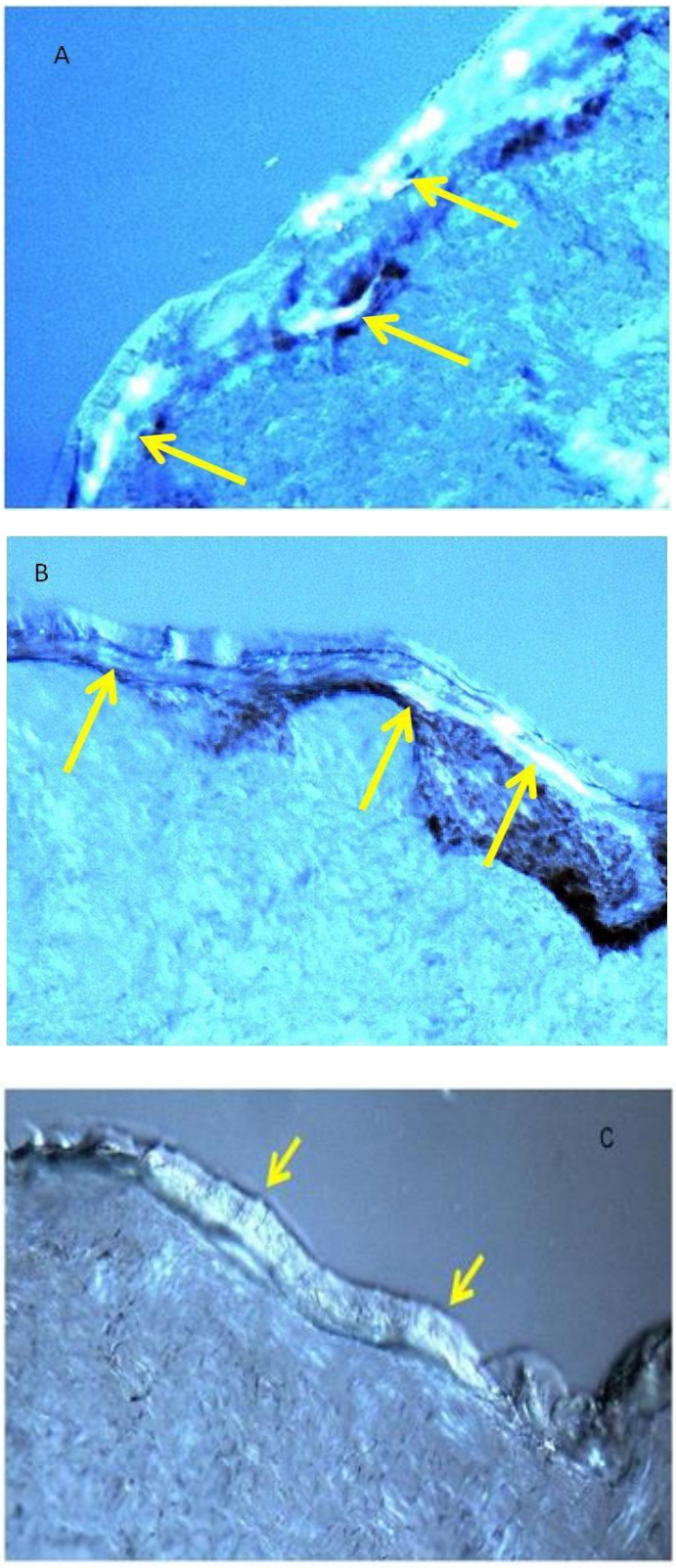


Figure 4: A plot showing the cumulative (additive) transit of the combined 50kDa/800 kDa HA into the skin via tape strips collected from the surface of the skin specimen in contact with the HA cream for an incubation duration of 1 h. This plot is simply an additive representation of the data shown in Figure 3.

2) Histological Results

For histological examination and visual indication of transit, the vesicles were loaded with trypan blue dye. Cream samples were tested in identical conditions as used in the tape stripping tests, i.e. biopsies were taken from skin specimens after 1 h incubation and frozen sectioned. The results showed visible transit of the dye into the skin, as shown in Figure 5.



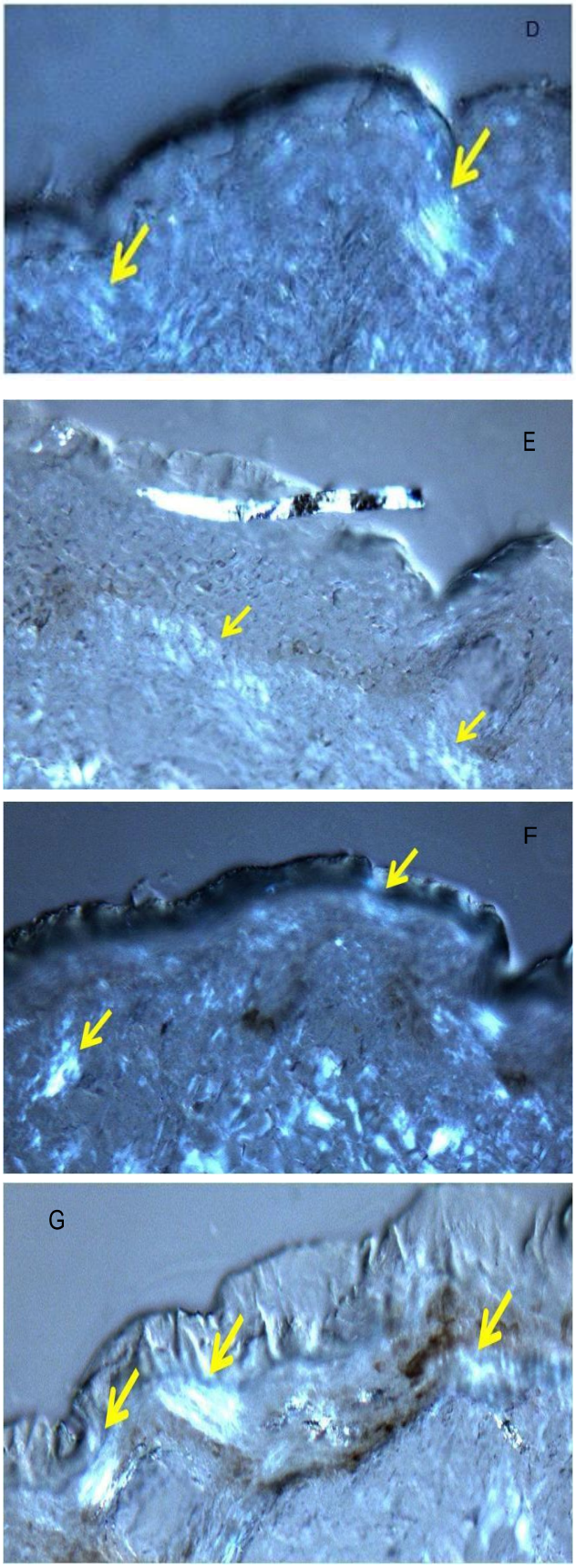


Figure 5: Histological illustration showing the transit of the vesicle-infused trypan blue dye into the skin specimen when using A) 50 kDa, B) 800 kDa HA, C) 5% Argireline, D) 2% Beta Glucan, E) 3% Tocopheryl Acetate, F) 3% Ascorbyl Palmitate and G) 5% Niacinimide. The yellow indicators show the presence of the dye, microscopically. Both images are represented at 10X magnification.

The activity of the individual constituents to transit and take residence in the dermis is mainly owing to the use of a specifically formulated amphiphilic block copolymer that allows for self-assembly into a three-dimensional spherical vesicle structure or a nanorod-like elastic vesicle structure. This specific vesicle structure is now shown to efficiently carry a variety of specific zeta charged cargos to skin cells.

Serum 1: This contains a hexapeptide-8 that is directed to the SNAP25 protein. The hexapeptide-8, a neurotransmitter peptide, mimics the N-terminal end of the SNAP-25 protein that inhibits the soluble N-ethyl- maleimide-sensitive factor attachment protein receptor (SNARE) complex formation". In figure 5C, it appears that the tagged hexapeptide has transited the stratum corneum and saturated the epidermis and infiltrated into the dermis.

Serum 2: This contains an oat beta-glucan that been fairly well established in skin moisturizing and has a history of healing minor wounds and burns. Figure 5D confirms that the molecules are small enough to penetrate the stratum corneum, epidermis and reached the dermis.

Serum 3: This contains a Tocopheryl Acetate (Vitamin E) created using an ester of Acetic Acid and Tocopherol being used as an alternative to pure Tocopherol (or undiluted Vitamin E). Tocopheryl acetate has photo-protective properties which can help protect skin against ultraviolet radiation. Figure 5E confirms that the tagged tocopheryl acetate component of the hydrating serum has transited and taken residence in the stratum corneum, epidermis and dermis.

Serum 4: This contains Ascorbyl Palmitate (Vitamin C) created using an ester combination of ascorbic acid and palmitic acid to form a fatty acid vitamin C to enhance its solubility in both fat and water. A major role of vitamin C is in manufacturing collagen. Ascorbyl palmitate is also an effective free radical-scavenging antioxidant. It also acts synergistically with vitamin E, helping to regenerate the vitamin E radical on a constant basis. Figure 5F confirms that the ascorbyl palmitate has transited the stratum corneum in significant quantities and has taken residence in the epidermal and dermal layers of skin.

Serum 5: This contains Niacinimide, (Vitamin B3) has been described as improving skin's elasticity, dramatically enhance its barrier function, help erase discolorations, and revive skin's healthy tone and texture. It has also been shown to increase ceramide and free fatty acid levels in skin, prevent skin from losing water content, and stimulate microcirculation in the dermis. Figure 5G clearly shows that the niacinimide has transited the stratum corneum and significant quantities have infiltrated the epidermis and dermal layers of skin tissue.

3) Skin Permeation Results

A common tissue donor was used with five independent site applications of the host pad tested on tissue specimens of 500 µm thickness through an incubation period of 24 hours with various sampling points in between. Both, the 50

kDa and the 800 kDa creams showed transit into the skin specimens at this incubation point (Figure 6). The 50 kDa samples showed greater transit into the skin when compared with the 800 kDa samples. There was no visible initial permeation for the first few hours, but that does not account for the amount of HA retained within the skin graft prior to diffusion into the receptor chamber.

The data shown in figure 6 denotes the permeation of the HA through 500 µm over a duration of 24 hours. It does not account for the amount of active material retained within the skin specimen. Following the 24 hour incubation duration, the samples were processed for retention (see methods for details) and results for retention as well as overall uptake (retention + permeation) are shown in Table 1. The 50 kDa samples produced greater transit when compared with the 800 kDa samples by a factor of greater than 2X. It can be speculated that the lower molecular weight HA may provide for an immediate effect onset whereas the larger molecular weight HA will have a slower release sustained mechanism of action. All results presented are normalized as detailed in the methods section.

Finally, it is visible from these results that the amount of HA retained within the skin graft, superficially, is magnitudes greater than that permeated past the skin graft and into the receptor chamber of the Franz cell setup. This indicates that under real time in-vivo conditions, the HA is expected to stay localized to the area under contact with the host material rather than diffuse away laterally or longitudinally. The implication of this is that the active ingredients will remain localized to the anatomical area of interest and focal application.

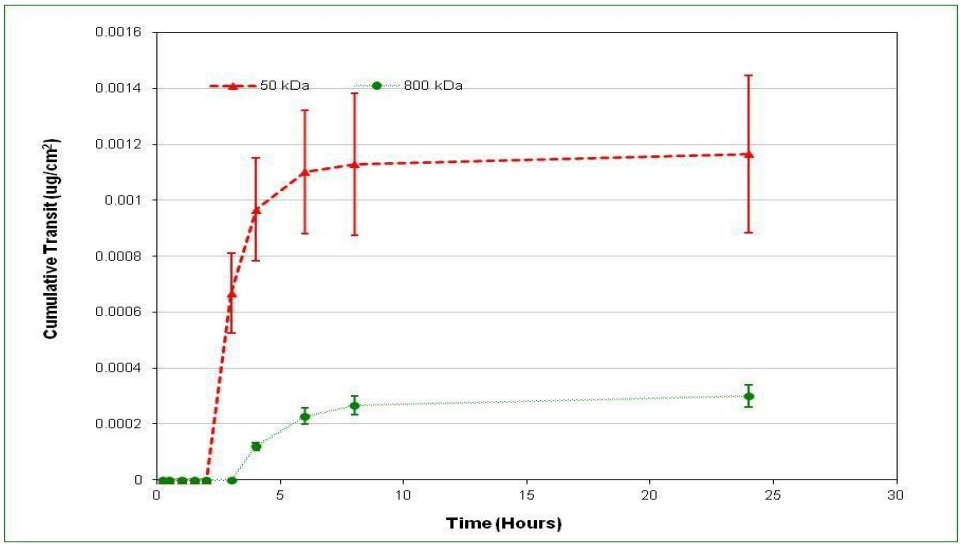


Figure 6: A plot showing transit of the HA into the skin grafts that were 500 µm in thickness. A greater transit is evident in the 50 kDa samples. There is a significantly visible transit in the 800 kDa samples.

Table III. Quantified data of transit of the HA into the skin grafts that were 500 µm in thickness. Approximately 3× greater transit in the 50 kDa samples was observed, but still a significantly visible transit was evident in the 800 kDa samples. The numbers shown above are reflective of the skin graft after removal of the stratum corneum by tape stripping. This explains why the numbers are a few orders of magnitude lower than those seen in the stratum corneum alone.

HA size	Permeation [μg]	Retention [μg]	Uptake [$\mu g/cm^2$]
50 kDa	0.0009±0.0002	237.83±41.83	302.81±53.26
800 kDa	0.0002±0.0000	105.47±10.09	134.28±12.85

These results indicate that the unique excipient used creates penetration into the stratum corneum and then interacts with structured lipids in the intercellular channels and releases them, thereby enhancing the penetration of hydrophilic actives through the channels. Additionally, the penetration enhancer penetrates into the intracellular matrix of the corneum that fluidizes the intracellular lipids and causes the reduction of diffusional resistance.

Also hypothesized is that a combination of both, the lower and higher molecular weight has the potential to offer a sustained release mechanism and effect. The hyaluronidases family of enzymes potentially has more of a rapid and short term effect on the smaller molecular weight HA than the higher molecular weight. Also, the higher molecular weight HA will continue to absorb water over a longer period of time, thereby providing a dual effect of short and long term sustained release.

These results and supporting data are in line with both Fick's First and Second Laws: wherein, the First Law relates to the diffusive flux to the concentration under the assumption of steady state. It further postulates that the flux goes from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient (spatial derivative). Fick's Second Law predicts how diffusion causes the concentration to change with time:

B. In-vivo studies

1) Post-treatment protocol

At Day 30 following fractional laser resurfacing, 100% of investigators agreed that the amount of erythema, edema, and pain all decreased through the trial and were substantially reduced by Day 30 with the statement "The healing is taking place more quickly compared with my standard post-treatment protocol." (64% strongly agreed; 36% agreed.) Ninety-one percent (73% strongly agreed; 18% agreed) that they would use the product again, and 100% agreed (64% strongly agreed; 36% agreed) that their patients were satisfied with the experience of using the serum. Patients were also uniformly in agreement with how their face was healing and by the amount of erythema (81% strongly agreed; 19% agreed), as well as the absence of pain (100% satisfied or strongly satisfied). Ninety-one percent agreed or strongly agreed that the serum was easy to use.

2) Deep hydrating cream protocol

Post-facial:

In the post-facial protocol, by Day 7 (the endpoint for the deep hydrating serum protocols), 81% of the investigators analyses reported that they would use the product again. Of the patients, 68% reported that they would use the product again; and 62% said they felt the serum added value to the procedure.

Lip and crow's feet:

By Day 7 of the lip and crow's feet protocol, 60% of investigators analyses agreed or strongly agreed that patients' fine lines appeared approved, and 94% of the procedure analyses reported that they would use the product again. Of the patients in the protocol, 65% reported that they would use the product again, and 52% agreed that the serum added value to the procedure.

Microdermabrasion:

At Day 7 following the microdermabrasion protocol, 51% of investigators analyses agreed or strongly agreed that patients' fine lines and wrinkles appeared to have improved and (91%) of analyses agreed or strongly agreed that erythema resolved more quickly than without the use of the cream. Nearly all (97%) of investigators analyses reported that they would use the product again. Patients reported similar satisfaction with the appearance of fine lines and wrinkles: 48% agreed or strongly agreed that their lines and wrinkles appeared better than baseline at Day 7. In addition, 65% said they would use the product again, and 44% reported that the cream added value to the procedure.

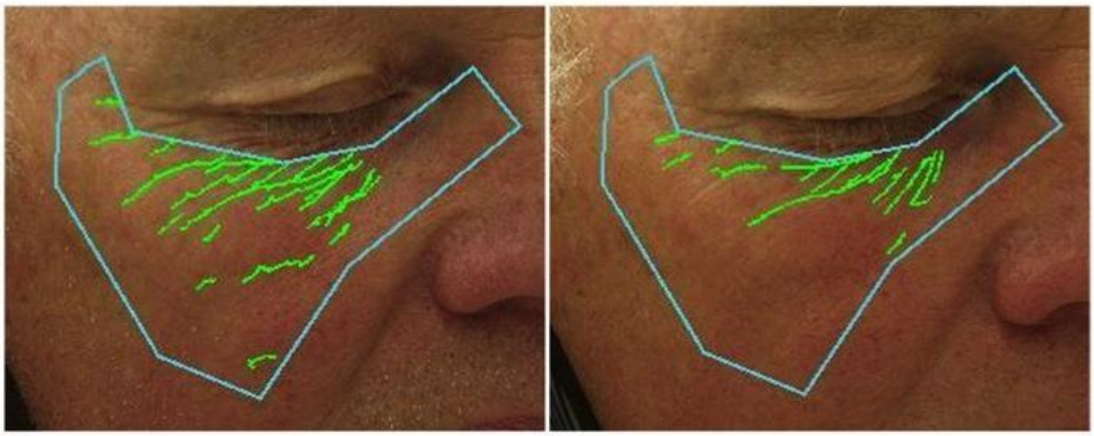


Figure 7: Photo of Subject 1 before the application of serums containing the precursor excipient and after the application treatment. A 28% reduction of fine lines was measured by VisiaTM camera system after 24 hours.

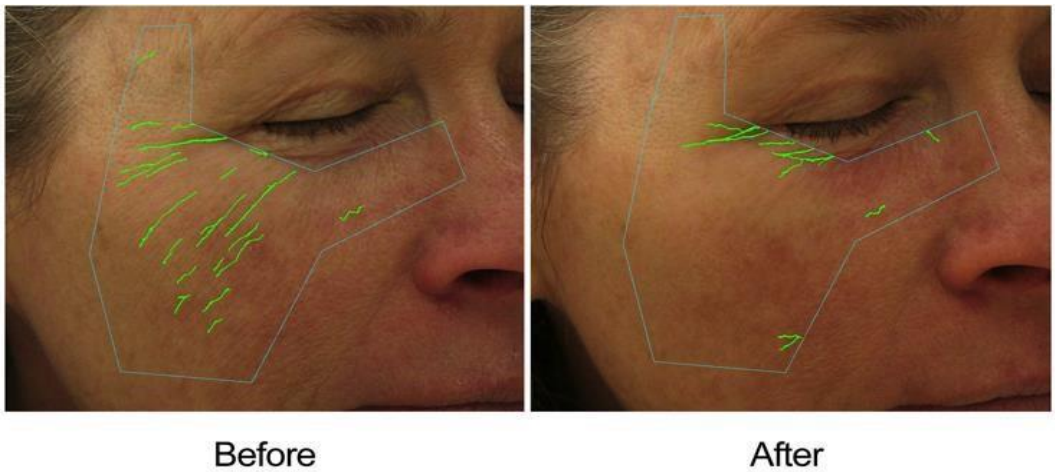


Figure 8: Photo of Subject 2 before the application of serums containing the precursor excipient and after the application treatment. A 22% reduction of fine lines was measured by VisiaTM camera system after 24 hours.

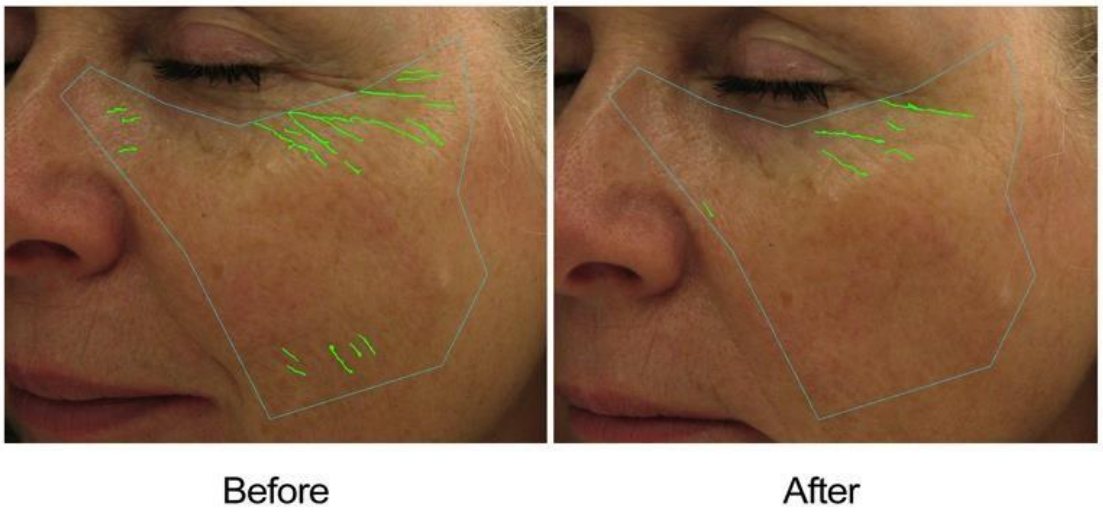


Figure 9: Photo of Subject 3 before the application of serums containing the precursor excipient and after the application treatment. A 20% reduction of fine lines was measured by VisiaTM camera system after 24 hours.

When used in the above aesthetic procedures, the HA infusion serums, lotions, and creams were well tolerated with no serious adverse events, no investigational device related adverse events and was well tolerated by study subjects. Both investigators and subjects rated the serums, lotions, and creams as easy to use with beneficial outcome measures in the areas of erythema, reduction of fine lines and wrinkles, reduction of post procedure pain, edema and redness. In addition, the study subjects had a significant degree of satisfaction in the performance of the serums, lotions, and creams, as indicated by the high response to the question of whether the investigational material host added value to the procedure.

IV. CONCLUSION

The stratum corneum and epidermis are effective barriers to the transit of endogenous materials into the skin. Various invasive and noninvasive modalities have been used to enhance the transit of active molecules, small and large in molecular weight. This is the first report that demonstrates the transit of a large molecule, hyaluronic acid, as well as certain additional active molecules such as argireline, beta glucan, tocopheryl acetate, ascorbyl palmitate, and niacinimide into the skin without the use of a treatment modality. The proprietary formulation of vesicles within the host serums, lotions, and creams allow the transit of HA into the skin, which helps with collagen remodeling and skin plumping (akin to injectable fillers).

The in-vivo studies suggested that the proprietary vesicle formulation incorporated into serums, lotions, and creams could be successfully used as a transepidermal delivery vehicle of hydrophilic and lipophilic actives and were well tolerated with no serios adverse effects. The creams were easy to use with beneficial outcome measures in the areas of erythema, reduction of fine lines and wrinkles, reduction of post procedure pain, edema and redness.

The novel technology of the Dynamic Infusion Technology products allows the delivery of hyaluronic acid (HA) and other constituents to cross the epidermal barrier of the intact stratum corneum and enhance the skin for the correction of a wide range of aesthetic skin concerns and dermatoses..

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Informed consent was obtained from all in vivo subject participants. All in vivo work was performed under the review of an approved IRB (Institutional Review Board) protocol. On Wednesday, August 12, 2015, select members of the Institutional Review Board of Concordia Clinical Research met to review the study protocol entitled “A demonstration of

the cutaneous Penetration of a Novel Hyaluronic Acid Serum: STUDY NUMBER DSC-64-15" under the conditions of an expedited review in accordance with paragraph 56.110, page 261 of the Federal register dated April 1, 1996, Part 56" The protocol aimed for the inclusion of representative human populations by sex, age and ethnicity.

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