**Introduction:** The stratum corneum is the skin’s chief barrier to prevent the transit of various exogenous materials into the viable epidermis and dermis. The stratum corneum is predominantly comprised of corneocytes (flattened cornified cells), with little to no water content, in the range of approximately 15%. Therefore, the dominant route for the transit of molecules into the skin is transfollicular liposomal based formulations. In addition to this, there is also a size exclusion criterion that applies to the transit of molecules into the skin. The stratum corneum with retained barrier function won’t allow transit of molecules or materials of beyond a certain size in order to maintain its function. Therefore, the entry of a relatively large molecule, Hyaluronic Acid (HA), for instance has historically been found to be extremely difficult.

Photoaging is one of the most common aging mechanisms known in skin conditions. Among various common conditions are solar elastosis, pigmentary dyschromia, lentigines, rhytids, etc. collectively culminating as chronological and environmental aging.

For reduction and repair of these changes, nonsurgical options include chemical peels and chemicals with minor irritant properties (e.g., topical retinoids, salicylic acid, and alpha-hydroxy acids), are 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.

For photoaged skin, for example, topical retinoids are an effective and common nonsurgical treatment. Topical retinoids work by decreasing 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. However, retinoids are commonly associated with adverse events (AEs) when 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.

Some traditional delivery mechanisms such as percutaneous injections (as in the case of dermal fillers), micro-needling (as in mesotherapy), iontophoresis, transdermal patches and liposomal formulations have been demonstrated to show efficacy of application. More recent advances in the space 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 the enhancement of transit of either small or lipophilic molecules.

In addition to delivery mechanisms, the type of molecule being transited into the skin is of critical importance. Traditional techniques, being primed for the enhancement of lipophilic molecules, don’t necessarily aid in the
allowance of hydrophilic molecules. This is especially true when the target area is not the stratum corneum or epidermis, but rather the dermis where critical aging structures, such as elastin and collagen, structurally reside. The reason for this is that the dermis is primarily comprised of water and therefore naturally is more conducive to the rapid uptake of water based molecules.

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

The study being presented takes a proprietary, interstice enhanced, biocellulose mask that also comprises penetration enhancers for HA and looks to assess the transit of the HA into human skin specimens. In addition to looking at a large molecule, this study also touches upon some key active ingredients that are smaller in size but yet pertinent to improvement in skin efficacy and turnaround.

**MATERIALS AND METHODS: In-Vitro**

**Materials:** Biocellulose masques containing HA with MW 50 kDa and 800 kDa, , Argirerline, Beta Glucan, Tocopheryl Acetate, Ascorbyl Palmitate and Niacinamide, were fabricated by Polyremedy, Inc., (Castro Valley, CA) were the primary components in this study. Tissue-Tek® O.C.T Compound was ordered from Sakura Finetek USA, Inc. HPLC grade water and Acetonitrile chemicals were ordered from VWR International, Inc. All other chemicals used in this study, not specifically identified, were also ordered 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 in-vitro proof of concept studies.

**HA Masque Fabrication:** A masque substrate was manufactured from a bio-(bacterial) cellulose. Gluconacetobacter xylinus (=Acetobacter xylinum, ATCC 10245) was purchased from the American Type Culture Collection and grown in 10g/l Backtopeptone (Difco), 10 g/l yeast extract (Fisher), 4mM KH$_2$PO$_4$ (Sigma) and 20 g/l D-glucose dissolved in deionized water (DI) The pH of the medium was adjusted to 5.1-5.2. The fermentation technique used yielded a robust method for the production of BC with predictable properties. Bacterial cellulose (BC, also known as microbial or bio cellulose, BC or MC) is one of the most abundant biodegradable materials in nature and has been the topic of extensive investigations in macromolecular chemistry.

The BC of the present formulation has a high aspect ratio with a diameter of 20-100 nm. As a result, the selected BC has a very high area per unit mass. This property, combined with its high hydrophilic nature, results in a very high liquid loading capacity. The fibrous structure of the aforementioned BC consists of a three-dimensional non-woven network of nanofibrils, sharing the same chemical structure as plant cellulose, which is held together by inter- and intra- fibrular hydrogen bonding resulting in a never-dry hydrogel state with high strength and high water retention. The BC is a gel containing 99% percent water by weight, mainly due to its amorphous structure. Helenis et al. implanted BC of the above mentioned method and formulation subcutaneously into rats and evaluated the implants with respect to chronic inflammation, foreign body responses, cell ingrowth, and angiogenesis through histology, immunohistochemistry, and electron microscopy. There were no macroscopic signs of inflammation around the implants: No fibrotic capsule or giant cells were present. Fibroblasts infiltrated BC, which was well integrated into the host tissue and did not elicit any chronic inflammatory reactions (Helenius et all, 2006)

A precursor of hyaluronic acid in solution was prepared by using amphiphilic polymers to form a micelle structure in an aqueous solution since the water solubility of their hydrophilic moiety greatly differs from that of their hydrophobic moiety. In the aqueous solution, the micelle has a unique core-shell structure wherein the hydrophobic moieties form an inner core and the hydrophilic moieties form an outer shell. The inner cores of such micelle is filled with water-insoluble hyaluronic acid (HA), thereafter which shows a greatly-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 micelle and to deliver HA onto a target depending on the surface properties thereof. Using this method of micelle construction, we are able to provide a method of delivering HA Stratum Corneum transit by using a micelle that consists of a shell region comprising hyaluronic acid (HA) and a core region comprising a water-insoluble peptide with a terminal amine peptide is bound to the hyaluronic acid. This provides a
delivery composition comprising a micelle 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 micelle. The delivery composition, the method of producing a HA-loaded, hyaluronic acid-conjugate micelle, and the method by using the micelle is where the biocompatible, biodegradable hyaluronic acid-peptide conjugate micelle 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 Hyaluronic Acid precursor and its host serum of fatty acid esters, polypeptides, polysaccharides, anti-oxidants and polymers was combined to effect a serum that is hydrated into the bio-cellulose delivery host during the masque fabrication process to create effectively Hyaluronic Acid infused fibers. These fibers are capable of either picking up greater quantities of exudates when in dry gel form, or donating greater quantities of fluids when in hydrated state, due to the inherent nature of hydrogels to have significantly high absorptive and hydrative capacities. Additionally, the masque could also act as a scaffold to facilitate migration and proliferation of cells in the wound and promote more rapid wound healing. The hyaluronic acid (HA) being used is a poly-anionic polysaccharide; particularly a thiol-derivatized hyaluronic acid. The HA is functionalized for E-Beam sterilization by adding one or more stabilizing excipients to the HA, or, in this case, by adding the HA to a formulation of stabilizing excipients. In this case we utilized the existing functionalizing precursor polymers in our fiber constituent bio-absorbable chemicals. Within the Polyethylene Oxide (PEO) constituent formulation used in the electrospinning of the dressing fiber there is contained Ethylene Vinyl Alcohol (EVAL). The EVAL content acts as an excipient to scavenge the effects of the E-Beam energy effectively stabilizing the polymer composition against the harmful effects of the sterilizing radiation. The normal effects to the HA, such as reduction in molecular weight, decreased solubility in aqueous solution, and alerted gelation times are mitigated. Further, the PEO component of the fiber solution acts on the HA as a covalent cross-linker, shielding the HA from the radiation effects by offering a host for the HA to adhere to.

Skin Preparation:

1. Upon arrival of fresh tissue, the skin specimens were placed between gauze pads that were soaked with 10X PBS (with 0.2% sodium azide).
2. The arrangement was then placed into a Ziploc bag and stored in a -80 °C freezer.
3. One night prior to expected experimentation, the frozen skin specimens were removed from -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.

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 (8mL capacity) with the PBS solution (pH 7.4) with 0.2% sodium azide (w/v).
4. Each specimen was then be 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 tested and the objective of the study.

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 gently 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 residual remained.
4. Using tweezers, scotch tape was applied on the specimen with the sticky side on the stratum corneum and rapidly removed.
5. Note: an untreated (no test material) control sample was also processed and analyzed in identical conditions as the test samples.
6. A total of 11 such tape applications were applied, the first of which was discarded due 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 with centrifugation at 12,000 rpm for 10 minutes at 4°C.
8. The supernatant solution was then drawn out of each tube/container, filtered and analyzed with an HPLC system for the amount of active ingredient retained in the skin specimen under test.
9. Averages of all samples tested were taken and recorded.

**Skin Permeation Method:**

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

1. Each piece was visually inspected for any defects, and any samples that appeared compromised were discarded.
2. The receptor chamber was filled (8mL capacity) with the PBS solution (pH 7.4) with 0.2% sodium azide (w/v).
3. 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.
4. 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.
5. 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.
6. 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 and 6 hours, 8 hours and 24 hours.
7. The aliquots were filtered and analyzed using reversed phase HPLC.
8. After the 24-hour permeation test was complete, the skin graft under test was washed with 5% soap solution.
9. Then a biopsy punch (6mm) 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).
10. This biopsy punch was diced using a scalpel and then homogenized for about 60 seconds in 10 seconds pulses followed by centrifugation (4 °C) at 10,000 rpm for 10 minutes.
11. 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.
12. Total uptake was calculated as the sum of the normalized cumulative permeation and the normalized retention in each sample.
13. Averages of all samples tested were taken and recorded.

**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 $V_e$.
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.
**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, where \( Y \) = Absorbance (mAU) and \( X \) = Mass (ug). 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, where \( t \) = sampling time interval and \( n \) = index number of the skin permeation system.

**Analysis Template:**

At each time interval, \( V_e = 1\text{mL} \) sample solution was extracted from each permeation cell, and \( V_r = 1\text{mL} \) 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:

\[
X_{1,n} = \frac{Y - C}{m} \cdot \left\{ \frac{V_o D}{V_i} \right\}
\]

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

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

The permeation of the molecule under test per cm\(^2\) 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\(^2\)

Where \( A \) = area of skin in contact with molecule under test (1cm 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. Since the graft thickness was known to vary, the permeation amount was normalized to a 500 µm skin graft thickness by using:

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

Where \( t_m \) was the measured graft thickness and \( t_i \) was the ideal graft thickness which was 500 µm. Averages of the samples were taken in order to determine the mean and standard deviation for permeation. Permeation ratio and ratio standard deviation could then be calculated. At the end of the permeation testing period (24 hours) a 6mm 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:
\[ Z_{t,n} = \frac{Y - C}{m} \left( \frac{V_c D}{V_i} \right) \]

Where \( V_c \) = volume of supernatant in 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 to the ideal mass for a 500 µm graft thickness. This was calculated by:

\[ R_{t,n} = \left( \frac{Y - C}{m} \right) \left( \frac{V_c D}{V_i} \right) \left( \frac{A_N}{A_B} \right) \left( \frac{M_i}{M_m} \right) \]

Where \( A_N \) = normalized area of skin in contact with 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 are taken in order to determine the mean and standard deviation for retention. Retention ratio and ratio standard deviation were also calculated. Normalized uptake of the molecule under test was calculated by using:

\[ U_{t,n} = X_{24\text{hours},n} + R_{24\text{hours},n} \text{ in mass of the molecule uptaken by the skin specimen in contact with the test solution.} \]

Uptake in mass per unit area was further calculated by:

\[ (U_{t,n} = X_{24\text{hours},n} + R_{24\text{hours},n})/A \]

Where \( A \) = area of skin in contact with molecule under test. Averages of the samples were taken in order to determine the mean and standard deviation for uptake enhancement. Uptake ratio and ratio standard deviation were then calculated.

**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 thickness 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 being tested (50 and 800 kDa) there were also tests carried out for individual constituents delivered through the following serums:

- Serum 1: 5% Argirerline
- Serum 2: 2% Beta Glucan
- Serum 3: 3% Tocopheryl Acetate
- Serum 4: 3% Ascorbyl Palmitate
- Serum 5: 5% Niacinimide

**RESULTS & DISCUSSION**

The preponderance of studies was carried out with 800kDa molecular weight hyaluronic acid (HA). This was done to have uniformity in gauging the changes in uptake through penetration enhancer and excipient modifications. Some defining studies were carried out to discern difference in performance in uptake of 50kDa versus 800kDa molecular weight HA. Further, a distinct transit evaluation of a combined content serum having both 50kDa and 800kDa HA was performed. Furthermore, histological testing was carried out on samples exposed to additional individual active
ingredients, presented within the embodiment of the ensuing results. Note, quantitative measurements were only carried out 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 host masks were tested on different areas of the tissue specimens through an incubation period of 1h. Both, the 50 kDa and the 800 kDa masks 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 mask 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 where samples contained only one molecular weighted form of HA. The test results for individual transit and cumulative transit are shown in figures 3 and 4 respectively. To be noted in these results was that despite the visible quantifiable transit of HA into the skin, the two isotopes were indistinguishable traces from one another.

![Figure 1](image.png)

Figure 1. plot showing transit of the HA into the skin via tape strips collected from the surface of the skin specimen in contact with the HA mask for an incubation duration of 1h. Note greater transit in the 50 kDa samples, while still significantly visible transit with the 800 kDa samples.
Figure 2. plot showing cumulative (additive) transit of the HA into the skin via tape strips collected from the surface of the skin specimen in contact with the HA mask for an incubation duration of 1h. Note greater transit in the 50 kDa samples, while still significantly visible transit with the 800 kDa samples. This plot is simply an additive representation of the data shown in figure 1.

Figure 3. plot showing 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 mask for an incubation duration of 1h.
2. **Histological Results**

For histological examination and visual indication of transit, the micelles were loaded with trypan blue dye. Mask samples were tested in identical conditions as used in the tape stripping tests, i.e. biopsies were taken from skin specimens after 1h incubation and frozen sectioned. Results showed visible transit of the dye into the skin as can be seen from figure 5.
Figure 5. Histological illustration showing transit of the micelle infused trypan blue dye into the skin specimen when using a) 50 kDa, b) 800 kDa HA, c) 5% Argirline, d) 2% Beta Glucan, e) 3% Tocopheryl Acetate, f) 3% Ascorbyl Palmitate and g) 5% Niacinimide masks. 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 due to the use of a specifically formulated amphiphilic block copolymer that allows for self-assembly into a three-dimensional spherical micelle structure or a nanorod-like micelle structure. This specific micelle 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 epidermal and dermal layers of skin tissue.

3. Skin Permeation Results

A common tissue donor was used with five independent site applications of the host masks 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 masks showed transit into the skin specimens at this incubation point (Figure 6). Also noted from the results was that the 50 kDa samples showed greater transit into the skin when compared with the 800 kDa samples. Also note that there is no visible initial permeation for the first few hours, but that does not account for the amount of HA retained still within the skin graft prior to diffusion into the receptor chamber.

Also of note here is that the data shown in figure 6 denotes the permeation of the HA through a 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. Also of note is that 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 while 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 mask.
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. plot showing transit of the HA into the skin grafts that were 500 µm in thickness. Note greater transit in the 50 kDa samples, while still significantly visible transit with the 800 kDa samples.

<table>
<thead>
<tr>
<th>Transit</th>
<th>50 kDa</th>
<th>800 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeation (µg)</td>
<td>0.0009±0.0002</td>
<td>0.0002±0.0000</td>
</tr>
<tr>
<td>Retention (µg)</td>
<td>237.83±41.83</td>
<td>105.47±10.09</td>
</tr>
<tr>
<td>Uptake (µg/cm²)</td>
<td>302.81±53.26</td>
<td>134.28±12.85</td>
</tr>
</tbody>
</table>

Table 1. table showing quantified data of transit of the HA into the skin grafts that were 500 µm in thickness. Note approximately 3X greater transit in the 50 kDa samples, while still significantly visible transit with the 800 kDa samples. Note that the numbers shown above are reflective of the skin graft after removal of the stratum corneum via tape stripping. It explains why the numbers are a few magnitudes lower than those seen in the stratum corneum alone.

These results indicate that the unique excipient being 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.
CONCLUSION

The stratum corneum and epidermis are effective barriers to the transit of endogenous materials into the skin. Various invasive and non-invasive modalities have been used to enhance the transit of actives, small and large in molecular weight. This is the first report we know of that shows the transit of a large molecule, Hyaluronic acid, as well as certain additional active molecules such as Argirerline, Beta Glucan, Tocopheryl Acetate, Ascorbyl Palmitate and Niacinimide into the skin without the use of a treatment modality. The proprietary formulation of micelles within the host masks allow the transit of HA into the skin wherefore it can be hypothesized that this will aid with collagen remodeling and skin plumping (akin to injectable fillers). While further studies in-vivo are warranted, the results suggest that the proprietary micelle formulation incorporated into biocellulose host masks could be successfully used as transepidermal delivery vehicle of hydrophilic and lipophilic actives.

The 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). And, where Fick's Second Law predicts how diffusion causes the concentration to change with time:

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5. Guidance document for the conduct of skin absorption studies, OECD Series on testing and assessment, Number 28, 2004
6. OECD Guideline for testing of chemicals, In-vitro skin corrosion: Human skin model test, April 2004

Clinical Trials

Two products, a post-treatment masque and a deep hydrating masque, 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 masque as an adjunctive agent in ablative or fractional ablative laser resurfacing. The other 3 protocols examined the deep hydrating lip and Crow’s Feet masques as an adjunctive agent for cosmetic facial procedures, the deep hydrating masque as an adjunctive agent in microdermabrasion. Study objectives, methods, and results for each of the protocols are reported below.

Post-Treatment Masque Protocol

The primary study objective of this protocol was to determine the effectiveness of the post-treatment masque, 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 masque, 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, edema, etc. 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 VISIA™ complexion analysis system or equally high-resolution photography. Photographs included a full frontal view, a 45-degree 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 one post-treatment masque to the face and instructed the patient to leave the mask in place for 24 hours. The patient was given 3 deep hydrating masques and instructed to apply 1 mask per day for at least 1 hour, 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.

Results

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 to 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 mask. 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 mask was easy to use.

Deep Hydrating Masque Protocols

The protocols using the deep hydrating masque had two study objectives: The primary objective was to determine the effectiveness of the facial mask 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 VISIA™ complexion analysis system or equally high-resolution photography. Photographs included a full frontal view, a 45-degree 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 pre-treatment assessment. Next, the investigator applied one Deep Hydrating mask for 45 min (30 min for microdermabrasion). For the lip and crow’s feet protocol, patients were given 2 sets of masks and instructed to apply one set of masks overnight for the next 2 nights. For microdermabrasion, patients were given 2 masks and instructed to apply 1 mask per day, for 45 min, over the next 2 days. See Figures 1,2,3. Inclusion and exclusion criteria are shown below:
Table 1: Inclusion and Exclusion Requirements: Post-Treatment Masque Protocol

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Subjects who meet all of the following criteria may be included in the study:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Females age 18 years or older</td>
</tr>
<tr>
<td>2.</td>
<td>Able and willing to provide written acknowledgment of participation</td>
</tr>
<tr>
<td>3.</td>
<td>Able to apply mask reliably, as recommended by the provider, either by self or with available assistance</td>
</tr>
<tr>
<td>4.</td>
<td>Able and willing to maintain patient log for reporting results</td>
</tr>
</tbody>
</table>

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<tr>
<th>Exclusion Criteria</th>
<th>Subjects who meet any of the following criteria will be excluded from the study:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Have received surgical or nonsurgical cosmetic procedures (including facials) at any time during the 4 weeks prior to initiation of the study</td>
</tr>
<tr>
<td>2.</td>
<td>Are scheduled to receive surgical or nonsurgical cosmetic procedures at any time over the duration of study (30 days)</td>
</tr>
<tr>
<td>3.</td>
<td>Are pregnant, lactating, or planning to become pregnant</td>
</tr>
<tr>
<td>4.</td>
<td>Have an open or healing lesion, rash, or other irritation on the face</td>
</tr>
<tr>
<td>5.</td>
<td>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)</td>
</tr>
<tr>
<td>6.</td>
<td>Have severe active facial acne</td>
</tr>
<tr>
<td>7.</td>
<td>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</td>
</tr>
</tbody>
</table>
### Table 2: Inclusion and Exclusion Requirements: Deep Hydrating Masque

<table>
<thead>
<tr>
<th><strong>Inclusion Criteria</strong></th>
<th>Subjects who meet all of the following criteria may be included in the study:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. Females age 18 years or older</td>
</tr>
<tr>
<td></td>
<td>2. Able and willing to provide written acknowledgment of participation</td>
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<tr>
<td></td>
<td>3. Able to apply mask reliably, as recommended by the provider, either by self or</td>
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<td></td>
<td>with available assistance</td>
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<td>4. Able and willing to maintain patient log for reporting results</td>
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<tr>
<td></td>
<td>at any time during the 4 weeks prior to initiation of the study</td>
</tr>
<tr>
<td></td>
<td>2. Are scheduled to receive surgical or nonsurgical cosmetic procedures at any</td>
</tr>
<tr>
<td></td>
<td>time over the duration of study (7 days)</td>
</tr>
<tr>
<td></td>
<td>3. Are pregnant, lactating, or planning to become pregnant</td>
</tr>
<tr>
<td></td>
<td>4. Have an open or healing lesion, rash, or other irritation on the face</td>
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<td></td>
<td>5. Have or have had a skin disorder that may confound measurement of</td>
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**Results**

**Post-facial.** In the post-facial protocol, by Day 7 (the endpoint for the deep hydrating masks 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 mask 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 masks 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 mask. 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 mask added value to the procedure.
Discussion

When used in the above aesthetic procedures, the HA infusion mask was 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 mask 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 mask, as indicated by the high response to the question of whether the investigational device (mask) added value to the procedure.

References


Summary of Technology

The novel technology of the Cellulation™ 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. HA is used as a primary ingredient because it plays a pivotal 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 agent.

HA molecules can absorb up to 3,000 times their own weight in water and, thus, have an important role as a hydrating agent as well. In wound healing, HA serves two important functions: First, HA provides a temporary structure in the early stages of the wound; 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. 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.

Because the stratum corneum is predominantly comprised of corneocytes, with little to no water content, the stratum corneum functions as a barrier that generally will not allow larger molecules or materials to pass. Through stratum corneum transit and serum infusion testing, physician investigators have validated the ability of Cellulation™ technology to deliver 50 kDa and 800 kDa HA through the stratum corneum, with additional proliferation into the epidermis. The Cellulation™ technology described here is novel in that it allows large and small molecules to pass into the epidermis. The researchers have tested the technology’s delivery capabilities with a wide range of molecule sizes and demonstrated that Cellulation™ can not only take them across the stratum corneum but also control how deep into the skin the active ingredients are deposited.
Figure 1: 28% reduction of fine lines as measured by Visia™ camera system at 24 hours.

Figure 2: 22% reduction in fine lines as measured by Visia™ camera system at 24 hours.
Figure 3: 20% reduction in fine lines as measured by Visia™ camera system at 24 hours.