Breakthrough on hyaluronic acid penetration inside hair fibres and keratin interaction for a smoothing effect explained

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Abstract

Background: The interaction between Hyaluronic acid (HA) and keratin has only been described in the *stratum corneum* and no evidence of a penetration and an interaction inside the hair has been established yet.

Methods: Low molecular weight hyaluronic acid (LMW at 1%), high molecular weight HA (HMW at 1%), or an optimized blend between low and high molecular weight HA (HA-Blend at 1%) were used. Hair locks were irradiated or not with UVs and then washed with the different shampoos described above. A different approach based on confocal Raman spectroscopy has been proposed to track their penetration through the human hair fibres. Then, the α-helix/β-sheet ratio of keratin was evaluated by Raman analysis to predict the smoothing effect. At the *ex vivo* level, we have measured hair strands length before and after eight hours in extreme conditions of humidity.

Results: The Raman analysis evidenced 5.9 times and 1.8 times more HA-Blend than the placebo into the hair cortex for the non-irradiated and the irradiated hair locks respectively. Regardless of the irradiation, the penetration of this optimal HA-Blend was significantly higher in comparison to the two other actives. The HA-Blend significantly decreased the α-helix/β-sheet ratio by -13% and the spontaneous frizzing by -11% *vs.* placebo.

Conclusion: Based on these results, confocal Raman spectroscopy can be considered as a powerful and non-invasive technique for investigating the penetration of active ingredient into the hair. We further demonstrated that an optimized formulation between different molecular weight HA is able to deeply penetrates the hair cortex to smooth the hair.

Keywords: Raman spectroscopy; hair penetration; hyaluronic acid; keratin conformation

Introduction.

Hair is a specialized derivative well known structure of the skin and it is ideally organized to protect human scalp. The external cuticle includes overlapping layers of scales and acts as a barrier to protect the inner structure; the medulla in the center surrounded by the cortex mainly made of keratin[1]. Keratin plays an important role for the physical and mechanical properties of the hair. At the molecular level, keratin is a helical protein and we distinguish two types of keratin fibres: type I, with acidic amino acid residues and type II, with basic amino acid residues. One strand of each type form together a coiled-coil dimer. These dimers coil together in an antiparallel tetramers known as protofilaments. Finally, these protofilaments interact together to form a single intermediate filament which is organized into larger micro-fibril and then into larger macro-fibril[2]. The keratin chains are found in an α-helix conformation, which are coiled by hydrogen bonds, ionic forces, Van Der Waals interactions and disulfide bonds. Disulfide bonds originate from sulfur-containing cystine bonds, which create a strong crosslink between adjacent chains and shape the hair. Indeed, the more hair keratin found in an α-helix conformation, the greater the curve in the protein chain and the more the hair fibre will curl. Conversely, a β-sheet conformation in the keratin decreases the formation of disulfide bonds and leads to a smoother hair fibre. Modifying disulfide bonds is a key mechanism of action in the haircare industry to shape and straight the hair. Current chemical agents such as alkaline reducing agent, thioglycolates or guanidine break and rearrange those bonds while damaging the hair and leading to lower hair strength[3]. Thus, safe active ingredients should be developed to smooth the hair without any damage for the hair, the consumer or the environment.

Hyaluronic acid (HA) is a key active ingredient in skincare products but its benefits for the hair has been poorly described. To date, the interaction between HA and keratin has only been described in the *stratum corneum* of the skin to enhance skin hydration[4]. No evidence of an interaction inside the hair has been established yet. Indeed, exploring penetration of such molecules through the hair fibres presents some challenges. Regarding the feasibility, different techniques, including confocal fluorescence microscopy, have been used for examining the penetration of tagged molecules but hair auto fluorescence compromise the

results[5]. Besides, due to the hair fibres cuticle, only low molecular weight molecules have been described, so far, to be able to penetrate the cortex[6]. Raman spectroscopy is a promising method to succeed this evaluation. This non-invasive technique detects the characteristic vibrational energy levels of a molecule and provides structural information. In confocal mode, it has the advantage of detecting information at various depths inside a tissue and a quantification is possible because of the linear correlation of molecules concentration and inelastic Raman scattering[7]. In a previous study, we have tracked the penetration of HA *ex vivo* through the skin using Raman spectroscopy[8].

In this work, we developed an efficient methodology to follow for the first time the penetration of HA through normal or UV-induced damaged hair in a non-invasive way. UVs irradiation damaged the hair by opening the cuticle to increase molecule's penetration. We then developed an optimized HA blend between low molecular weight HA and high molecular weight HA dedicated to haircare application based on optimal hair penetration. Once into the cortex, this optimal HA-Blend interact with keratin to change its conformation in favor of β-sheet conformation for a visible smoothing effect.

Materials and Methods.

HA penetration by Raman spectroscopy

Hair fibre preparation and treatment

In this study, we used 10 natural, undyed, light blond hair locks from a Caucasian donor in order to limit fluorescence interference during Raman spectroscopy measurements. Half of the hair locks were irradiated with both 20 J/cm² UVA and 0.6 J/cm² UVB. Then, the 10 hair locks were washed for 2 minutes with 500µL of shampoo containing 1% of LMW, HMW or HA-Blend, rinsed 3 times with 200mL of distilled water and dried 3 minutes with a hair dryer. The cycle of washing, rinsing and drying was repeat 3 times before analysis. The same formula without any active was used as placebo. Untreated hair locks were washed with distillate water.

Raman spectroscopy and confocal measurements (Z) on hair fibres

The Raman spectra were recorded using a near infrared confocal Raman micro-spectrometer (Labram, Horiba Jobin Yvon, Villeneuve d'Ascq, France). The set-up comprised an optical microscope (Olympus, BX41, France) coupled to a dispersive Raman spectrometer (Horiba Jobin Yvon, Villeneuve d'Ascq, France) and a charge-coupled device (CCD) detector. The excitation source was provided by a titanium–sapphire laser (Model 3900S, Spectra-Physics, France) generating a laser beam with a 785 nm wavelength and operating at 50 mW under the microscope objective, which is non-destructive for light blond hair samples and does not cause any thermal or photochemical degradation. Confocal Raman measurements were recorded using a 100x infra-red optimised objective (Olympus, France) operating in air with a numerical aperture of 0.8. To obtain axial Z profiles, the objective was mounted on a high-precision piezoelectric device (Physics instrument, Germany) which allowed vertical in-depth scanning by focusing the laser light on each depth point through the hair fibres. The spectral acquisitions were recorded all along the hair fibre with a scanning Zstep size of 3 µm, and for each spectrum, one accumulation of 30 s of laser exposure was taken. Data acquisition was performed using LabSpec 5 software (Horiba Jobin Yvon, Villeneuve d'Ascq, France).

Spectral data processing

The pre-processing of spectral data was performed using LabSpec 5 software (Horiba Jobin Yvon, Villeneuve d'Ascq, France). First, cosmic radiation was removed and all aberrant profiles were excluded from the database. Each remaining profile was subjected to corrections to clean up the Raman signal for the hair fibres. These corrections applied to the raw spectral data included the correction of spectral shifts, noise reduction using a 5-point average Savitzky–Golay smoothing filter, and baseline correction using a polynomial function of degree 5 to remove the fluorescence background. Three independent axial Z profiles were recorded for each hair sample.

Keratin conformation by Raman spectroscopy

Treatment of hair samples

The first sample was not treated and was packaged immediately in aluminum foil; this was the negative control for keratin α-helix conformation. The second sample was smoothed with an iron and then packaged; it served as the positive control for keratin β-sheet conformation. The three other samples were soaked in 200 mL of distilled water each and massaged with 0.5g of shampoo containing 3% of HA-Blend or just 0.5 g of the basic shampoo used as a placebo. Each sample was then rinsed three times in three baths of distilled water and wiped with paper before being dried with a hair dryer for 3 min. These samples were also packaged in aluminum foil and stored at room temperature before being subjected to Raman spectroscopy analysis with the same parameters than previously described. After the pre-processing steps were completed, the corrected average spectra from 30 µm were processed using self-coded software based on a hierarchical classification algorithm and Amide I band curve-fitting methods operating in the Matlab environment (The Math Works Inc., U.S.A.). The average spectra from axial z profiles of hair fibres were classified using Ward's clustering algorithm. This function calculates Euclidean distances between the spectra and groups them into clusters according to their similarities. This method of cluster analysis allows for the classification of hair fibres with a similar keratin structure.

Anti-frizz *ex vivo* analysis

The study was done on hair locks with different curly level. Ten hair locks per conditions were tested. At T0, 10 hair locks with different curl degree (from wavy to curly) were selected. Each hair lock was divided in equal parts (2 treated parts and one non-treated part) in order to have an equal distribution in term of curl degree. Photographs were realized before washing. Then hair locks were washed with shampoos containing 1.5% or 3% HA-blend, placebo shampoo or water for non-treated hair locks. All hair locks were dried in open air. Photographs were done after treatment and drying. The hair locks were smoothed with a hair straightener. Photographs were done immediately after hair smoothing. Then hair locks were put in a room under extreme conditions of humidity (Relative humidity 80% ± 10% RH). Photographs of hair locks were performed after 8 hours in extreme conditions. Photographs were analysed using Photoshop® to study anti-frizz effect by the measure of hair strands length before and after four and eight hours in extreme conditions of humidity.

Statistical analysis

The results that followed a parametric distribution were subjected to statistical analysis using Student's t-test, while those that did not follow a parametric distribution were subjected to statistical analysis using the Mann–Whitney test. Significant P values are presented in figures as follows: *p value < 0.05; **p value < 0.01; *** p value < 0.001.

Results.

HA penetration through the hair fibres

We tracked the penetration of several active ingredients through irradiated or non-irradiated hair fibres using Raman spectroscopy. The cuticle was defined between 0µm and 12µm and the cortex between 12µm et 42µm. Regarding the non-irradiated hair locks (Figure 1) the penetration of the active inside the cuticle was significantly higher than the placebo and increased by a fold x6.9, x4.9 and x6.9 for the HMW, LMW and HA-Bend shampoos respectively. For the penetration inside the cortex, the HA-Blend was found significantly 5.9 times more than the placebo. The penetration inside the cortex of the HA-Blend was also significant in comparison to the other active shampoos.

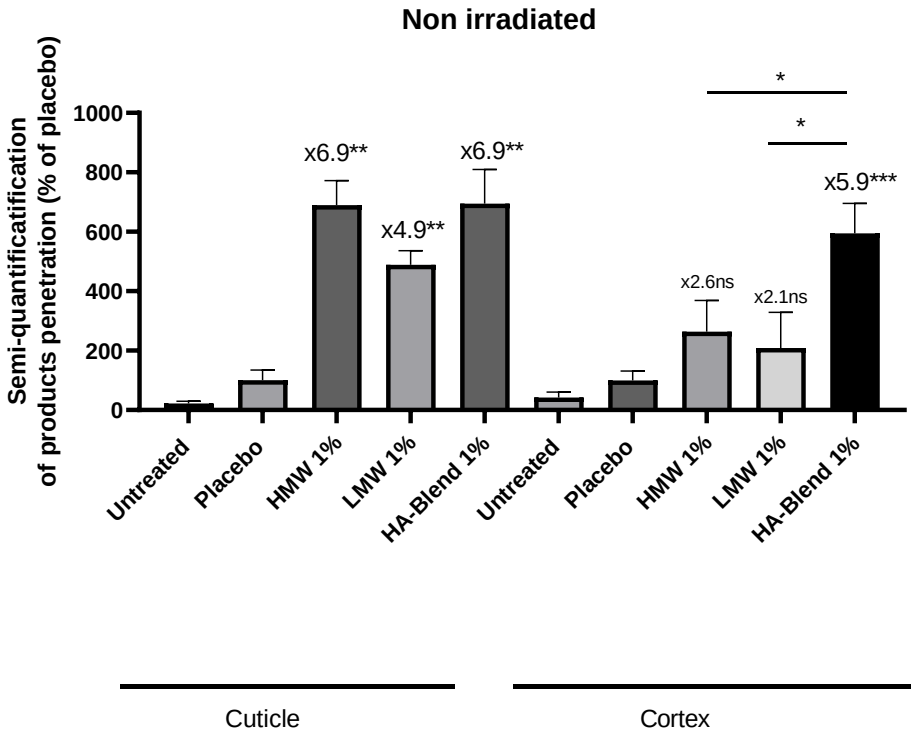

Figure 1. Semi-quantification of the penetration of LMW 1%, HMW 1% and HA-Blend 1% shampoos through non-irradiated hair fibre. The penetration was analyzed both through the cuticle and the cortex. *p<0.05 ***p<0.001.

About the irradiated hair fibers with UVA and UVB, the penetration inside the cuticle of the HA-Blend was significantly reduced by x0.1 times in comparison to the placebo whereas its

quantity inside the cortex was significantly increased by x1.8 times. The quantity of the HA blend at 1% inside the cortex of the irradiated fibres was significantly higher than HMW at 1% and LWM at 1% (Figure 2).

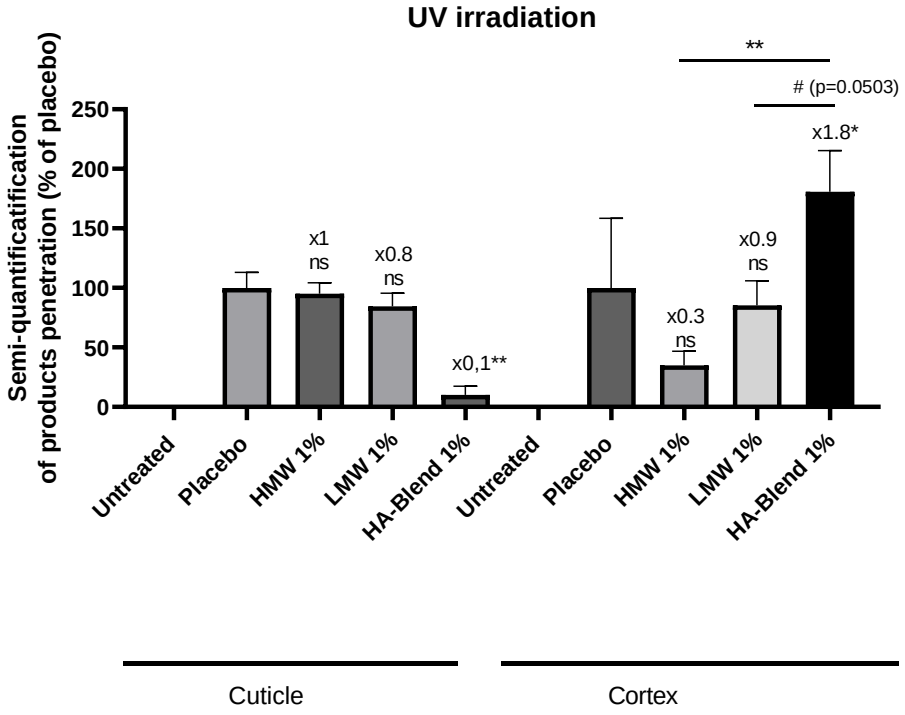

Figure 2. Semi-quantification of the penetration of LMW 1%, HMW 1% and HA-Blend 1% shampoos through irradiated hair fibre with UVA and UVB. The penetration was analyzed both through the cuticle and the cortex. # $p<0.1$ *$p<0.05$ ** $p<0.01$.

Keratin conformation with the ratio α-helix/β-sheet

Once we proved that our optimal HA-Blend was able to penetrate the cortex in higher amount than the other HA types, we analyzed its interaction with keratin inside the cortex by the analysis of α-helix/β-sheet ratio. This study was done on non-damaged hair and shown that the ratio was significantly decreased by 7% after treatment with 1.5% of HA-Blend in comparison to the untreated hair (Figure 3).

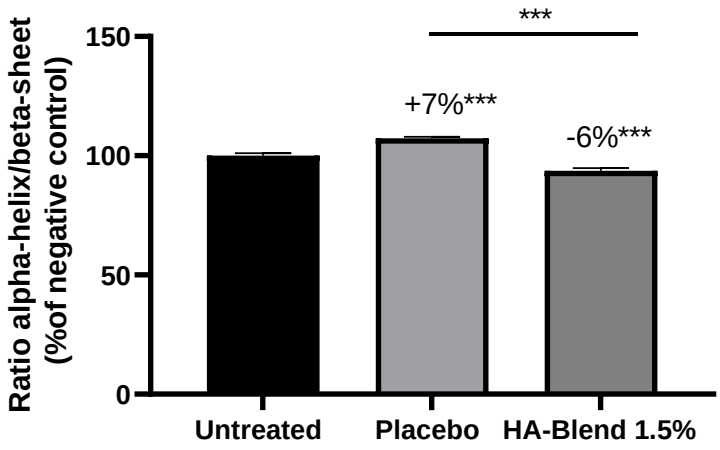

Figure 3. Evaluation of the α-helix/β-sheet keratin ratio after hair treatment with 1.5% of HA-Blend. *** $p<0.001$.

Anti-frizz study

Finally, after elucidating a possible straightening benefit of the HA-Blend linked to its direct interaction with keratin and the decrease of the α-helix/β-sheet keratin ratio, we compared the anti-frizz effect. Hair locks were washed with HA-Blend at 1.5% or 3% shampoos. After straightening with a hair straightener, hair locks were put in extreme humidity condition. After 8 hours of incubation, pictures were taken and smoothing effect was calculated based on the hair length variation (Table 1). HA-Blend at 3% significantly smoothed the hair by 11% in comparison to the placebo shampoo for a visible effect (Figure 4).

Table 1. Comparison of anti-frizz effect of shampoos applied on hair locks after extreme humidity conditions for 8 hours.

Condition and parameters	Smoothing percentage (mean±SD) and *p* value	Δ(%) versus placebo
Untreated	69±3 ($p<0.001$)	
Placebo	83±4 ($p<0.01$)	
HA-Blend 1.5%	87±2 ($p<0.01$)	4% (ns)
HA-Blend 3%	94±2 ($p<0.01$)	11% ($p<0.05$)

Figure 4. Macrophotographies illustrating the anti-frizz effect of HA-Blend in comparison to the placebo.

Discussion.

For the very first time, we established an innovative study to prove the penetration of hyaluronic acid inside the hair fibre in rinse-off condition. Irradiation with both UVA and UVB damaged the hair and opened the cuticle to enhance product penetration. That allows us to compare and understand the penetration of our active into normal or damaged hair locks. Regarding the non-irradiated hair locks, penetration of the three active shampoos (LMW, HMW and HA-Blend) inside the cuticle was significantly higher than the placebo without a significant difference between the products. Whereas the penetration of the HA-Blend was significantly higher inside the cortex than the placebo, the LMW and the HMW. About the damaged hair locks with UVs irradiation, the penetration of the HA-Blend inside the cuticle was significantly lower and the penetration into the cortex was significantly higher than the placebo and the two other active shampoos. After UV-induced damages, the placebo is able to penetrates the cuticle and it has been previously described that hydrophilic compound such as HA has a higher binding force to protein such as keratin[9]. We could hypothesize that once the HA-Blend penetrates the cuticle in damaged condition, this optimal formulation will be attracted by the keratin inside the cortex and slight amount remain on the cuticle. Interestingly, even in the non-irradiated condition, only the HA-Blend was significantly able to deeply penetrate the cortex due to an optimal formulation between low and high molecular weight HA attracted by keratin. We also demonstrated that this optimized HA-Blend decreased α-helix conformation and promoted β-sheet keratin conformation in order to smooth the hair. This data was already highlighted in a previous study with further analysis showing that this HA-Blend decrease the disulfide bound of the keratin and thus the α-helix/β-sheet ratio[10]. We have now established that the change of keratin conformation is due to the penetration of the HA-Blend and a direct interaction between HA and keratin. At the *ex vivo* level, the anti-frizz action of the HA-Blend was confirmed with a significant smoothing effect by 11% in comparison to the placebo and a visible benefit. Moreover, it has been described that interaction between HA and keratin in the skin is involved in hydration and skin barrier integrity, we could hypothesized that same interaction in the hair could have benefits on hair structure and further studies should be carried on[4].

Conclusion.

Based on these results, Confocal Raman spectroscopy can be considered as a powerful and non-invasive technique for investigating the penetration of hair cosmetic ingredients in human hair fibres. To the best of our knowledge, this study is the very first to prove and compares the penetration of different molecular weight hyaluronic acid inside hair fibres. In this work, we demonstrated that an optimized formulation between different molecular weight HA was able to deeply penetrate the hair cortex to interact with keratin and smooth the hair. This insight opens new doors for the haircare industry to understand the mechanism of action of active molecules for hair benefits.

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Conflict of Interest Statement. NONE.

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