Demonstrating permeation of an anti-ageing peptide into the *stratum corneum* using 3D OrbiSIMS

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Abstract

Background: Tracking and profiling the permeation of skincare active ingredients through the *stratum corneum* can be challenging, particularly if the chemistry of the ingredient is similar to the native chemistry of the skin. Here, the state-of-the-art 3D OrbiSIMS technique has been used to demonstrate the permeation of the biologically active anti-ageing peptide Palmitoyl Tripeptide-1 (Pal-GHK) into the *stratum corneum*.
Methods: A 3D OrbiSIMS approach was used to determine the presence and intensity of the Pal-GHK peptide *in vivo*, through analysis of tape strips collected from product treated and untreated human volar forearm *stratum corneum*, and *ex vivo*, using full thickness human skin in a Franz-type static diffusion cell set-up.
Results: The depth profile of Pal-GHK across 15 *stratum corneum* tape strips was determined, and further analysis of the data showed that Pal-GHK could be clearly detected in the 10th tape strip and beyond, demonstrating significant penetration ($p<0.05$, versus untreated control) to at least 10 layers into the skin surface. In addition, Franz-cell experiments demonstrated that the Pal-GHK molecular ion was clearly detected, with the ion intensity decreasing with increasing skin depth, reaching a baseline level at the *stratum corneum* – epidermal junction.
Conclusion: 3D OrbiSIMS can successfully identify and provide relative quantification of the molecular ion produced from low concentrations of Pal-GHK in the *stratum corneum*, despite the peptide's similarity to native skin components. This novel approach has merit in demonstrating effective permeation of low molecular weight peptides through the *stratum corneum*; data that has previously been challenging to attain.

Keywords: Permeation, Peptides, *Stratum corneum*, Mass Spectrometry, OrbiSIMS

Introduction

Crossing through the *stratum corneum* is the rate limiting step in the permeation of skincare active ingredients into the underlying epidermis and beyond. While numerous approaches can be employed to enhance the permeation of active ingredients, tracking and profiling the permeation of these ingredients through the *stratum corneum* can be challenging, particularly if the chemistry of the active ingredient is similar to that of the native chemistry of the skin. Tape strips have been used for many years as a methodology to sample layers of the *stratum corneum* and have been used to investigate the dermato-pharmacokinetics of topically applied substances *in vivo* [1, 2].

Palmitoyl Tripeptide-1 (Pal-GHK), a synthetic palmitoyl oligopeptide, is a biologically active anti-ageing peptide that is highly utilized in the cosmetic market [3, 4]. Commercial formulations containing this peptide have been shown to have anti-ageing benefits, stimulating the deposition of dermal fibrillin-1 microfibrils and reducing facial wrinkle clinical grade [5, 6]. Pal-GHK is composed of a peptide fragment found in the collagen-1 protein and is a key component of the active ingredient Matrixyl 3000^{TM}. Being native to the skin and chemically similar to peptides found in nearly 3000 different proteins in the skin, means it has previously been highly challenging to measure the permeation profile of the peptide without tagging it with a radioactive or fluorescent label that changes its molecular mass and penetration characteristics.

Preliminary research by the No7 Beauty Company and the University of Nottingham used time-of-flight secondary ion mass spectrometry (ToF-SIMS) to assess the permeation of active ingredients in both tape strips and *ex vivo* skin samples. This method has proven successful in measuring and visualising Vitamin C permeation in *ex vivo* skin samples [7] and chlorhexidine in both skin explants and tape strips [8]. However, ToF-SIMS lacks the sensitivity and mass resolving power to be able to conclusively identify low concentration peptide signals. Recently the more sophisticated 3D OrbiSIMS instrument has been used to determine depth profiles of native skin proteins such as collagen and keratin [9] and offered a potentially greater level of sensitivity for identification of low concentrations of peptide. The 3D OrbiSIMS was developed at the UK National Physical Laboratory (NPL) in collaboration with the University of Nottingham and other partners. It combines secondary ion mass spectrometry with the high mass-resolving power of an Orbitrap^{TM} mass analyser, facilitating *in situ* label free molecular analysis and the identification of organic species in complex solid samples, including biological tissues [10]. Here, a 3D OrbiSIMS approach was used to determine the Pal-GHK peptide content *in vivo* in tape strips collected from the human volar forearm *stratum corneum* and *ex vivo* in full thickness human skin explants.

Materials and Methods

For the *in vivo* study, twelve Caucasian subjects (11 female, 1 male; aged 30-55) were treated with three serum-type formulations containing <100 ppm Pal-GHK applied at 2mg/cm^2 to the volar forearms, with one area remaining untreated. All subjects gave informed consent to participate in the study. The study was performed in a constant environment of 22 ± 2 °C and 50 ± 5 % humidity and subjects were acclimatized for 15 minutes prior to product application. Four hours post-application, 15 sequential tape strips (22mm diameter) were collected from all sites using D-squame® tape strips (CuDerm). The D-Squame® pressure instrument was used to apply consistent pressure for 5 seconds before removing tape strips from the skin. Optical absorption of the tape strips was measured in triplicate at 850 nm to determine skin cell density. Previous work has shown that optical absorption correlates with protein content of the skin cells [11]. Tape strips were stored in Eppendorf tubes, immediately placed on ice after collection and then stored at 4°C until analysis. 3D OrbiSIMS was then used to determine the presence and intensity of the Pal-GHK peptide at 3 separate points on the tape strip. The molecular peak intensity was normalised against total ion content and an average of the triplicate values taken. The average molecular peak intensity was subsequently normalised against the average optical absorption of corresponding tape strips, therefore adjusting for inter-subject variability in the amount of *stratum corneum* captured in each tape strip.

For *ex vivo* analysis, full thickness human skin tissue was removed during cosmetic surgery from Caucasian female donors (aged 35-70). The explant was frozen immediately post-surgery, shipped and stored at – 20 °C prior to use. It was then defrosted, cut and mounted, dermal side down, in a Franz-type static diffusion cell set-up [12], with an exposed surface area of 1.1 cm^2. Infinite doses of formulations containing Pal-GHK at <100 ppm were applied to the donor chamber, with the explant exposed to the formulations for 4 hours in a water bath set to 36.5 °C. Sink conditions were maintained throughout the experiments. After 4 hours, the Franz cell was dismantled, excess formulation was removed with a dry sponge and the skin explant was wiped with a sponge soaked in Teepol Solution (3 % v/v). Next, the explant was dehydrated under vacuum at room temperature for 24 hours before 3D OrbiSIMS analysis.

3D OrbiSIMS analysis was performed on a Hybrid SIMS instrument (IONTOF GmbH) under the following conditions; A 20 KeV Ar_{3000}+ analysis beam with a diameter of 20μm was used as the primary ion source. Samples were analysed at ambient temperature across a 400 × 400μm area in positive polarity with sawtooth raster mode and a total crater size of 486 × 486μm. Duty cycle was set to 4.4% and cycle time to 200μs. Mass spectra were recorded at a resolution of 240,000 at m/z 200 in the mass range of 75 to 1,125 m/z. Both data acquisition and the subsequent data processing were performed using SurfaceLab 7 software (IONTOF GmbH).

Results

Throughout the analysis, the Pal-GHK molecular ion, $C_{30}H_{55}N_6O_5+$ (Fig.1), was used as the diagnostic marker, having first been confirmed to be present and absent in the formulations and unused blank tape strips respectively.

The depth profile of Pal-GHK across 15 *stratum corneum* tape strips was determined for 3 different serum-type formulations each containing <100 ppm Pal-GHK. All 15 tape strips were analysed for 3 subjects to determine the entire penetration profile of the peptide. To visualize the data, tape strips were grouped in sequential sets of three strips (i.e. 1-3, 4-6, 7-9, 10-12, 13-15) and the peptide ion intensities totalled for each set of three. The data showed that the Pal-GHK peptide ion signal was detected right through the 15 tape strips (Fig 2) for all 3 formulations. Based on the results of these 3 subjects, 5 tape strips centred on tape strip 10 (tape strips 8-12) were chosen for further analysis.

Peptide intensities were determined for the selected 5 tape strips across 12 subjects for each of the serum-type formulations. Analysis of the data showed that Pal-GHK could be clearly detected in tape strips 8-12 (Fig. 3), and while there was some variability between the formulations, there was a significant difference versus the untreated control for all samples (Student's t test, $p<0.05$). There is some analytical noise detected in the untreated samples for this ion's mass position, but this has been confirmed as background noise. The data was further interrogated to focus on tape strip layers 10-12 of the 12 subjects (Fig. 4). The peptide intensity drops with increasing depth (Fig. 2) and as expected, there was some biological variability in the peptide concentration in the *stratum corneum* samples. However, there was considerable Pal-GHK peptide detected in tape strips 10-12 for all 3 formulations, with over 65% of subjects having detectable peptide at tape strip 10 or beyond (Student's t test, $p<0.05$ vs untreated control). Therefore, this data shows that Pal-GHK from all 3 formulations permeates to at least 10 surface layers deep in the majority of subjects.

A formulation containing <100 ppm Pal-GHK was applied to human *ex vivo* skin in a Franz-cell chamber. The tissue was left for 4 hours before being analysed with the 3D OrbiSIMS. The ionising beam of the OrbiSIMS sputters the surface of the tissue, with increased sputter time corresponding to increased depth into the tissue. The data demonstrated that the Pal-GHK molecular ion was clearly detected, with the ion intensity decreasing with increasing skin depth, reaching a baseline level at the *stratum corneum* – epidermal junction (Fig. 5). The *stratum corneum* – epidermal junction was located to a sputter time of approx. 4000s in previous work by tracking the secondary ion markers of phospholipids (data not shown). Phospholipids can be used as a marker of epidermal tissue as they are practically absent from the *stratum corneum* [13].

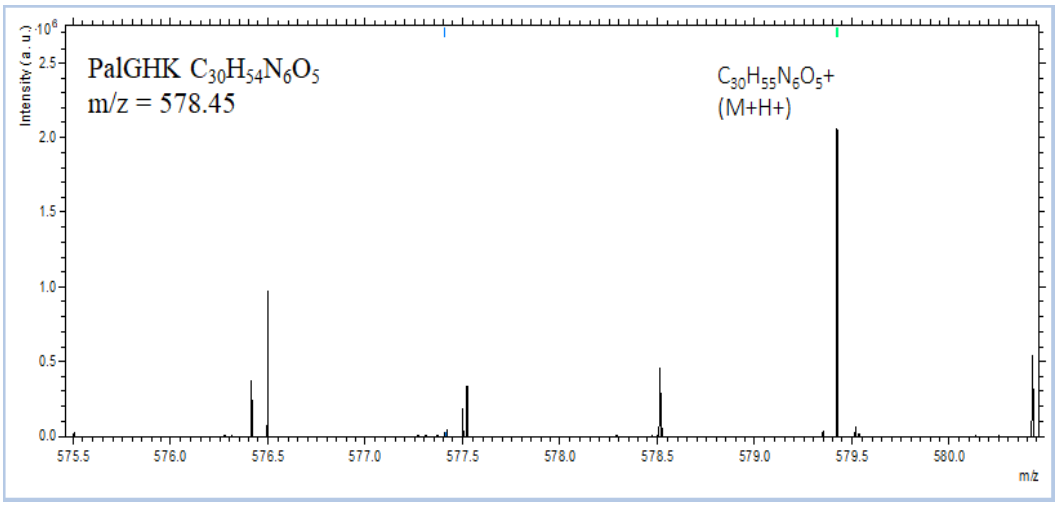

Fig 1: *3D OrbiSIMS spectrum identifying the Pal-GHK molecular ion.*

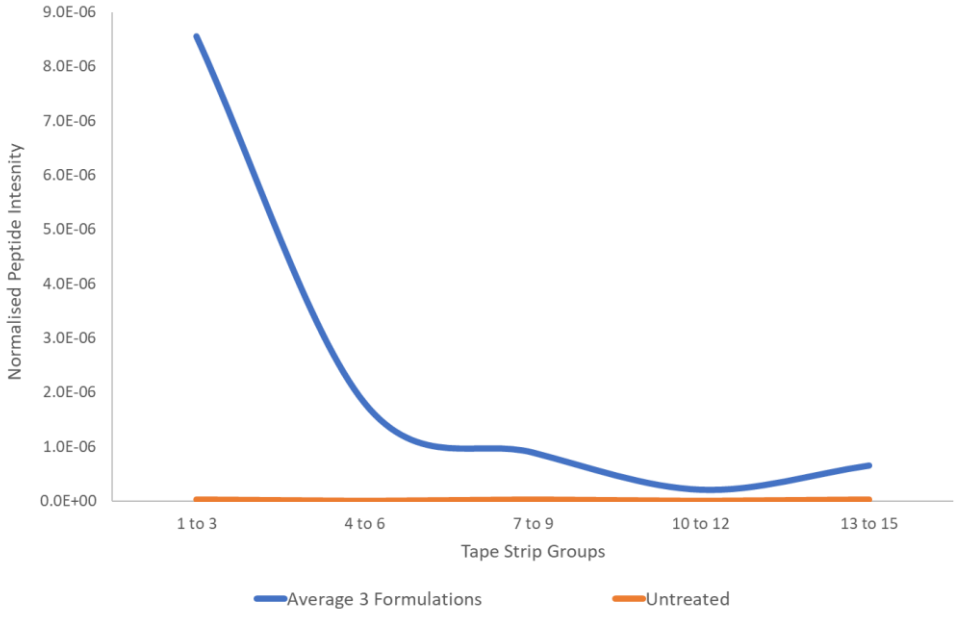

Fig 2: *Peptide Intensity of Pal-GHK Across Entire Depth Profile; Data shown is the totalled peptide intensities for each set of three tape strips, averaged across 3 serum-type formulations, compared to the untreated control, for 3 subjects. Tape Strips 8 to 12 were chosen for subsequent analysis.*

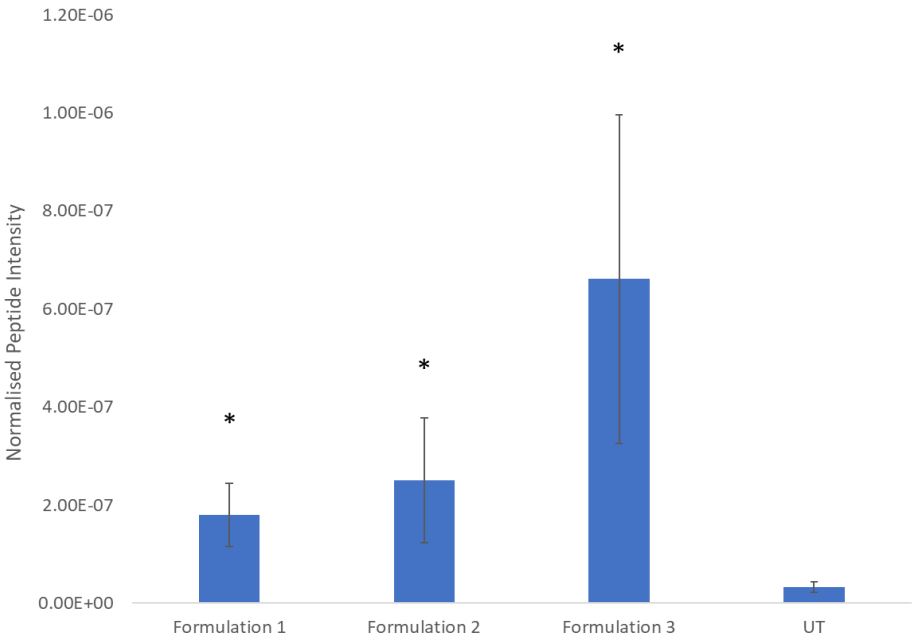

Fig 3: *Total Peptide Intensity in Tape Strips 8-12; Data shown is average total peptide intensity in tape strips 8-12 for n of 12, +/- SEM, for three formulations and the untreated control (UT), * p<0.05 v untreated.*

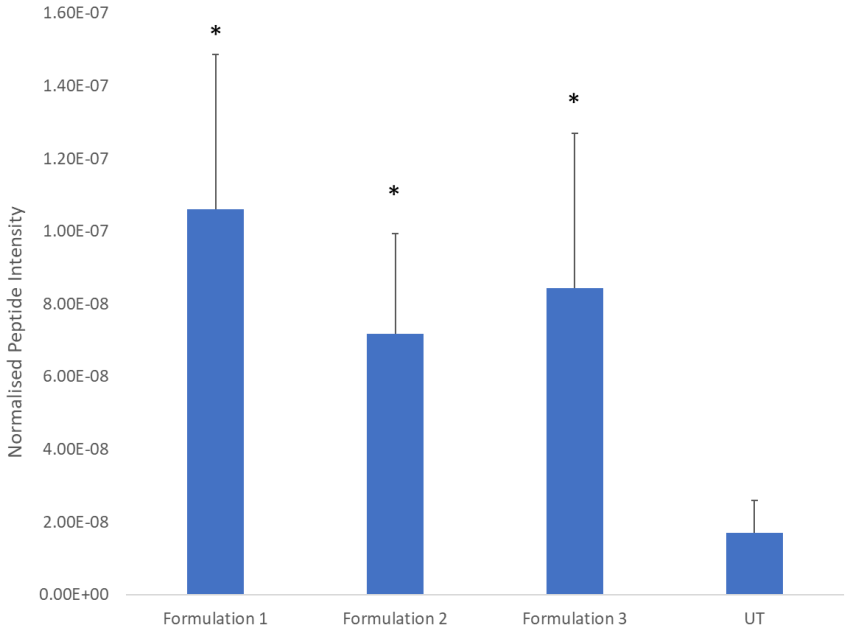

Fig 4: *Total Peptide Intensity in Tape Strips 10-12; Data shown is average total peptide intensity in tape strips 10-12 for n of 12, +/- SEM, for three formulations and the untreated control (UT), * p<0.05 v untreated, for >65% subjects in which peptide was detected in tape strip 10 or above*

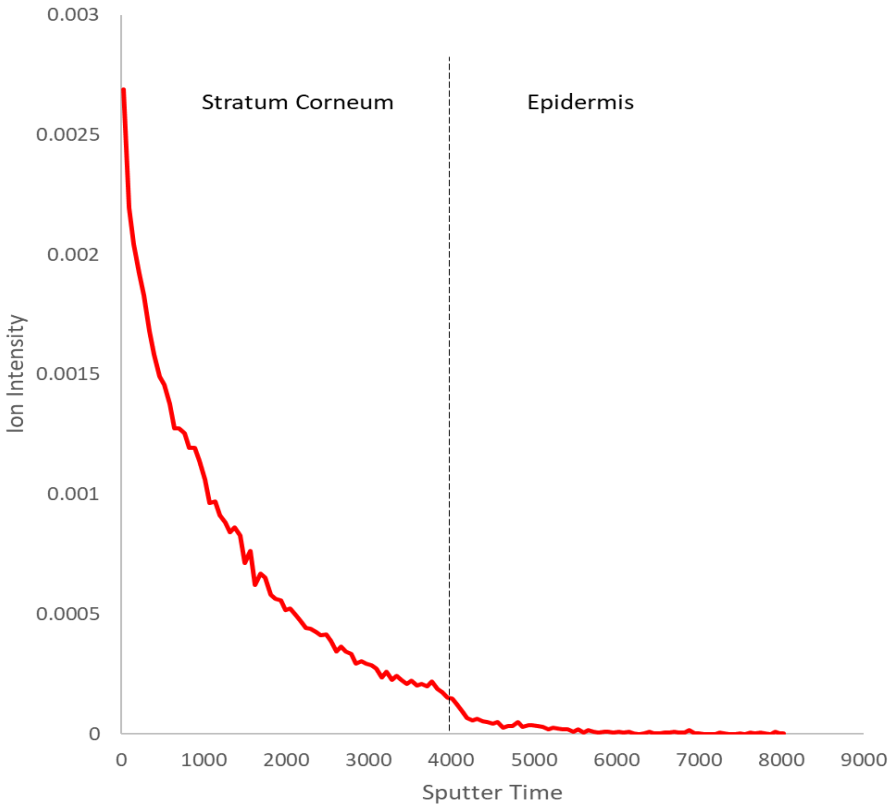

Fig 5: *Ex vivo Permeation of Pal-GHK; the stratum corneum-epidermal junction is marked with a dotted line.*

Discussion

Using a 3D OrbiSIMS profiling methodology we have been able to track the permeation of Pal-GHK, both *in vivo* through *stratum corneum* tape strip samples and through *ex vivo* human skin explants, at commercially relevant levels. The data shows that the Pal-GHK molecular ion decreases in intensity with increasing skin depth, with a similar pattern observed both *in vivo* and *ex vivo*. While a decrease in intensity with increasing skin depth was not unexpected, we were nevertheless able to clearly show permeation of the peptide through 15 tape stripped layers of the *stratum corneum* and demonstrate statistically significant penetration to at least 10 layers into the skin surface for all three formulations. These findings were supported by the *ex vivo* depth profile, where the Pal-GHK molecular ion was observed to decrease in intensity with increasing skin depth, reaching a baseline level at the *stratum corneum* – epidermal junction. Taken together, this data clearly shows that Pal-GHK can permeate through layers of the *stratum corneum*, the rate limiting step for the permeation of topical active ingredients. This provides support for its ability to reach deeper layers of the skin in order to elicit repair of structural proteins such as fibrillin-1 microfibrils at the dermal-epidermal junction and reduce the appearance of wrinkles.

In a recent study, Kawashima et al. have demonstrated the analysis of a similar collagen tripeptide in skin using ToF-SIMS [14]. However, despite using a solution that was 1000 times more concentrated than that used in this study, their results suggest that the molecular ion peak of the tripeptide was difficult to detect, even in the second layer of the *stratum corneum*, and multivariate analysis was needed to confirm its presence. Our analysis was conducted following application of a complex formulation with the active compound at levels <100ppm, and despite its low concentration within the skin and its chemical similarity to native skin components, we were able to clearly identify the Pal-GHK molecular ion ($C_{30}H_{55}N_6O_5+$) and assess its permeation as a function of skin depth.

Conclusion

Despite the low concentration of Pal-GHK within the skin and its chemical similarity to native skin components, this novel approach clearly identified the molecular ion and facilitated assessment of the permeation of the peptide as a function of skin depth; data that has previously been difficult to accurately attain. 3D OrbiSIMS has enormous potential to provide molecular information for skin research and further understanding of active ingredient delivery. Indeed, we have recently published further research where the 3D OrbiSIMS was used to characterise the complex chemistry of the skin, particularly that of the *stratum corneum* [15].

Acknowledgments

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Conflict of Interest Statement

MO'M, MJ and MB are employees of Walgreens Boots Alliance.

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