Enhanced vitamin C skin permeation from supramolecular hydrogels, illustrated using *in situ* ToF-SIMS 3D chemical profiling

Nichola J. Starr[a], Khuriah Abdul Hamid[a†], Judata Wibawa[b], Ian Marlow[b], Mike Bell[b], Luïsa Pérez-García[a], David A. Barrett[a], David J. Scurr[a*]

[a] Advanced Materials and Healthcare Technologies, School of Pharmacy, University of Nottingham, NG7 2RD, UK
[b] Walgreens Boots Alliance, Nottingham, NG90 1BS, UK
[†]Permanent address: Faculty of Pharmacy, Department of Pharmaceutics, Universiti Teknologi MARA Selangor, 42300 Puncak Alam, Malaysia.

***Corresponding author:** David.Scurr@nottingham.ac.uk

Abstract

Vitamin C (ascorbic acid) is a naturally occurring, powerful anti-oxidant with the potential to deliver numerous benefits to the skin when applied topically. However, topical use of this compound is currently restricted by an instability in traditional formulations and the delivery and eventual fate of precursor compounds has been largely unexplored. Time of flight secondary ion mass spectrometry (ToF-SIMS) is an emerging technique in the field of skin research and offers detailed chemical analysis, with high mass and spatial resolution, as well as profiling capabilities that allow analysis as a function of sample depth. This work demonstrates the successful use of ToF-SIMS to obtain, *in situ*, accurate 3D permeation profiles of both ascorbic acid and a popular precursor, ascorbyl glucoside, from *ex vivo* porcine skin. The significant permeation enhancing effect of a supramolecular gel formulation, produced from an amphiphilic gemini imidazolium-based surfactant, was also demonstrated for both compounds. Using ToF-SIMS, it was also possible to detect and track the breakdown of ascorbyl glucoside into ascorbic acid, elucidating the ability of the hydrogel formulation to preserve this important conversion until the targeted epidermal layer has been reached. This work demonstrates the potential of ToF-SIMS to provide 3D permeation profiles collected *in situ* from *ex vivo* tissue samples, offering detailed analysis on compound localisation and degradation. This type of analysis has significant advantages in the area of skin permeation, but can also be readily translated to other tissue types.

Keywords

Skin permeation, supramolecular hydrogel, permeation enhancer, vitamin C, ToF-SIMS, depth profiling.

1. Introduction

Vitamin C (ascorbic acid) is well established as a natural anti-oxidant (Bendich *et al.*, 1986). Topical application of this compound has shown success in both protecting against UV-induced oxidative stress (Darr *et al.*, 1992; Offord *et al.*, 2002) and offering regenerative properties, with a clinical improvement in the microstructure of aged skin linked to a stimulation of collagen synthesis (Humbert *et al.*, 2003; Murad *et al.*, 1983; Phillips *et al.*, 1994). These effects, coupled with the ability to combat hyperpigmentation (Farris, 2005), have resulted in ascorbic acid becoming a popular ingredient regularly employed in cosmetic products, especially anti-aging focused products. Topical ascorbic acid has also shown potential for clinical use, demonstrating both anti-inflammatory and wound healing properties (Farris, 2005). However, due to its proficiency as an antioxidant, it is readily oxidised, especially in aqueous-based systems. Formulation for topical application therefore typically results in rapid degradation of the compound, drastically reducing the stability and shelf life of the product.

A popular solution to this issue, especially in the cosmetics industry, is the use of precursor compounds. These are typically ester or ether derivatives that offer improved stability and are proposed to be enzymatically converted to ascorbic acid within the skin (Farris, 2005; Pullar *et al.*, 2017; Stamford, 2012). The clinical effects of several derivatives have been demonstrated, as reviewed by Farris (Farris, 2005). However, their permeation efficiencies and mechanisms of action are still largely unexplored, including their rate and extent of conversion into ascorbic acid (Pullar *et al.*, 2017; Stamford, 2012). These are all parameters that cannot be easily observed using the techniques typically applied for skin permeation analysis.

Novel delivery vehicles are an alternative option, either to stabilise ascorbic acid for topical delivery or to enhance the permeation of precursor compounds. Research focused on ascorbic acid delivery includes incorporating compounds into nanocarriers, such as liposomes

(Zhou *et al.*, 2014), and alternative formulation types, such as anhydrous suspensions (Heber *et al.*, 2006). Recent work in the field of topical delivery has demonstrated the success of a novel gelator, specifically an amphiphilic gemini imidazolium-based surfactant, to produce supramolecular hydrogels that are able to incorporate drug compounds and facilitate enhanced skin permeation (Limon *et al*., 2017; Limon *et al*., 2017, 2015; Rodrigues *et al*., 2014). Supramolecular gels are well suited to topical application as they are both soft and non-permanent. It has been demonstrated that these novel gel formulations are simple and quick to produce and are extremely versatile. To date, these novel gel formulations have been tested on a range of compounds with diverse molecular structures, most recently three pharmaceuticals currently employed in commercial formulations, triamcinolone acetonide, brimonidine tartrate and betamethasone 17-valerate (Limón *et al*., 2017). Limón *et al.* demonstrated that “drug release was up to 10 times faster and retention of the drug within the skin was up to 20 times more effective” when the gel formulation was applied vs. the commercial product (Limón *et al*., 2017). These gels therefore offer a promising prospect to improve localised skin delivery, but have yet to be explored for the delivery of popular cosmetic actives, such as ascorbic acid.

Understanding the skin permeation of exogenous compounds is an area of research that is essential to many industries. Whether the aim is to facilitate permeation (pharmaceutical or cosmetic products) or to prevent it (pesticides or pollutants), detailed chemical analysis is crucial. Information regarding the permeation depth of a compound into skin tissue is key for the assessment of targeted skin delivery into the epidermis or dermis. It is also useful to explore effects such as permeation enhancement and the location of compound degradation within the skin, whether unsolicited or by design. The industrial standard for assessing skin permeation is to conduct *in vitro* Franz cell diffusion experiments combined with chromatographic mass spectrometry (MS) analysis (Franz, 1975; Friend, 1992). While this method works well for assessing transdermal permeation, through routine analysis of the underlying receptor solution, acquiring a direct permeation profile for compounds residing within the skin tissue itself is more challenging. Tape stripping can be used to sample the *stratum corneum* (Celebi *et al.*, 2016) and horizontal cross-sectioning can obtain skin slices

from varying depths (Lapteva *et al.*, 2014), but chromatographic MS analysis of these samples relies on both efficient compound extraction and successful column isolation from co-extracted native components. In addition, these processes all require an extra preparation step and further manipulation of the tissue. Tape stripping has been shown to be subject to large variability, dependent on numerous parameters that have to be strictly controlled (reference). The separation of epidermal and dermal layers requires exposure to either heat or harsh chemicals. Cross sectioning requires prior embedding of the tissue and again exposure to exogenous chemicals, which Sjövall *et al.* has recently suggested can have a "compromising effect" on the localisation of lipids within a tissue ((Sjövall *et al*., 2018).

Recent advancements in mass spectrometry imaging (MSI) techniques have seen them rise in popularity for chemical analysis of biological samples, as reviewed in detail by Chughtai and Heeren (Chughtai and Heeren, 2010). These techniques offer a key advantage over chromatographic MS, through their ability to raster a sample surface and produce chemical ion maps, providing information on the spatial distribution of compounds across a surface. This is especially useful when assessing the localisation of either endogenous or exogenous compounds within biological material, such topical product delivery to the skin.

Time of flight secondary ion mass spectrometry (ToF-SIMS) is a type of MSI technique and requires no prior sample preparation, apart from a requirement for the sample to be placed under high vacuum. It can analyse a multitude of samples, from tissue sections (Gamble *et al*., 2015) to individual cells (Brison *et al*., 2011) and can conduct simultaneous analysis of all components within a sample. ToF-SIMS is therefore able to provide a rapid assessment of the chemistry within a biological sample, without the need for extensive method development to extract and separate components of interest, an aspect of chromatographic MS that can sometimes prove time-consuming. ToF-SIMS is unique as an MSI technique, as it has the ability to conduct both surface and depth profile analysis. As well as rastering across a surface, ToF-SIMS is able to sputter through a sample and concurrently collect MS information as a function of sample depth. This enables the production of 3D chemical profiles, without the need for the sample to be sectioned or deconstructred. One disadvantage of this technique is

that it only provides semi-quantitative information, providing a relative comparison of skin permeation rather than calibrated compound concentration.

Numerous studies have shown successful surface analysis of skin samples, both tape stripped stratum corneum (Holmes *et al*., 2017; Judd *et al*., 2013; Starr *et al*., 2016) and cross-sectioned ex vivo tissue (Kezutyte *et al*., 2013; Kubo *et al*., 2013; Monteiro-Riviere *et al*., 2011; Sjövall *et al*., 2014). The most recent publication regarding ToF-SIMS analysis of skin permeation, published in 2018 by Sjövall *et al*., highlights the significant advantage that can be provided by this technique (Sjövall *et al*., 2018). Using surface analysis of cross sectioned skin tissue to assess carvacrol permeation, Sjövall *et al*. were able to demonstrate a strong localisation of the compound within the dermal layer, which was in good agreement with gas chromatography MS data. However, the imaging capabilities of ToF-SIMS could also determine that the localisation within this layer did not exhibit a lateral uniformity, suggesting possible delivery via a follicular route.

Similar results have been achieved using surface analysis of cross sectioned skin tissue (Kezutyte *et al*., 2013; Kubo *et al*., 2013; Monteiro-Riviere *et al*., 2011; Sjövall *et al*., 2014) yet the profiling capabilities of this technique have been explored to a much lesser extent. Sjövall *et al*. state that one drawback to their study is that the “preparation of the cross-section samples is critical and can be problematic” (Sjövall *et al*., 2018). *In situ* depth profile analysis of skin tissue would circumvent the need for sectioning. Yet only one study to date has exploited these depth profiling capabilities to assess skin permeation, successfully demonstrating localisation of the common hapten, nickel, in order to further investigate its role in contact allergy (Per *et al*., 2017).

As previously discussed in detail by Judd *et al.* (Judd *et al.*, 2013), other imaging techniques have been applied to analyse skin as a function of depth, most notably confocal laser scanning microscopy (Alvarez-Román *et al.*, 2004a, 2004b; Gerger *et al.*, 2006; Rajadhyaksha *et al.*, 1995) and both stimulated Raman scattering microscopy and confocal Raman spectroscopy (Ashtikar *et al.*, 2013; Freudiger *et al.*, 2008; Pyatski *et al.*, 2016; Saar *et al.*,

2011). These all offer the ability to conduct 3D *in vivo* imaging of the skin. However, ultimately, they do not possess the chemical sensitivity and specificity offered by MS techniques. This makes it hard to distinguish between native skin components and certain exogenous compounds, especially cosmetic actives which are often based on naturally occurring compounds. Furthermore, assessment of compound metabolism is also difficult without the specificity provided by detailed MS analysis.

The aim of this work was to explore ToF-SIMS 3D profiling as a method to provide in situ skin permeation analysis of active compounds within ex vivo skin tissue. Specifically, to investigate the permeation enhancing effects offered by a supramolecular hydrogel formulation for the localised delivery of both ascorbic acid and a popular precursor, ascorbyl glucoside.

2. Experimental

2.1 Materials

Ethanol (ACS reagent grade) and phosphate buffered saline tablets were purchased from Fischer Scientific, UK. 1,3-bis[(3-octadecyl-1-imidazolio)methyl]benzene dibromide was synthesised at the University of Barcelona (Spain) according to the previously reported protocol (Rodrigues *et al*., 2014). Ascorbic acid and ascorbyl glucoside were purchased from DSM, Switzerland and DKSH, UK respectively. 10 % neutral buffered formalin, xylene (histological grade) and paraffin wax were purchased from Sigma Aldrich, UK.

2.2 Skin Sample Preparation

Porcine ears were obtained from animals aged 4-6 months, reared specifically for food. This ensured that removing the skin tissue was a secondary use of the animal. The ears were removed from the pig immediately after slaughter, prior to cleaning or hair removal. The skin was stored for a maximum of 24 hours at 4 °C, after which the tissue was washed with ultrapure water only and the hair trimmed with scissors. Using a scalpel, the outer-ear skin was detached from the underlying cartilage and any excess subcutaneous fat was removed. The skin was wrapped in aluminium foil and stored at -20 °C for a maximum of 3 months prior

to use. Full thickness skin sections (approximately 2mm) were used for both the cross sectioning and for the Franz cell permeation experiments.

The effect of freezing and thawing on the barrier function of *ex vivo* skin tissue has been largely debated. However, recent studies on *ex vivo* human tissue stored at both -20 °C (Jacques-Jamin *et al.*, 2017) and -85 °C (Barbero and Frasch, 2016) conditions observed no significant change in the permeation of several model compounds. The effect of freezing on skin metabolism is another issue that arises from the use of *ex vivo* skin tissue. It is largely assumed that previously frozen skin tissue is rendered metabolically inactive. However, several studies have shown that there is still some metabolic activity present in *ex vivo* skin that has been frozen and thawed (Hewitt *et al.*, 2000; Nicoli and Santi, 2007). It is important to note that all skin samples used in this study have been stored and prepared in an identical manner. It can therefore be assumed that any permeation differences observed between samples are genuine and not artefacts related to sample preparation.

2.3 Preparation of blank tissue cross sections

A 1 cm × 1 cm square of porcine skin was fixed in a 10 % (v/v) formalin solution for 20 minutes. After washing in phosphate buffered saline (PBS), the skin sample was then dehydrated using a series of ethanol solutions. The sample was soaked in each solution for 30 minutes, increasing in ethanol concentration from 25 - 100 % (v/v). The sample was then twice submerged in xylene (2 × 1 hour) and lastly twice in paraffin wax (2 × 1 hour). The dehydration and embedding process was conducted automatically using a tissue processor (Leica TP1020, Milton Keynes, UK). The embedded sample was then cut vertically into sections of approximately 10 µm using a microtome (Leica RM 2165 microtome, Leica Microsystems Ltd.) and mounted onto glass slides. Prior to ToF-SIMS surface analysis, the cross sections were paraffin-stripped in xylene and left to air dry.

2.4 Formulation Preparation

Both ascorbic acid and ascorbyl glucoside were prepared as phosphate buffered saline (PBS) solutions and supramolecular hydrogel formulations, both at 5% (w/v), see Table S1. The

gelator used was 1,3-bis[(3-octadecyl-1-imidazolio)methyl]benzene dibromide. To prepare the gel formulation, 5 mg of gelator was dissolved in 400 µL of ethanol. In a separate vial 50 mg of ascorbic acid/ascorbyl glucoside was dissolved in 600 µL of deionised water. The compound solution was then added to the gelator solution and stirred manually for 60 s. The mixture was then left undisturbed until gelation had occurred, which was confirmed using the inverted vial method (Rodrigues *et al.*, 2014). Supramolecular hydrogels produced using this gelator have shown to be "non-irritant" when applied *in vivo* (Limón *et al*., 2015).

2.5 Franz Cell Set-Up

Once defrosted, circular skin samples were cut and mounted, dermal side down, between a donor and receptor chamber in a Franz-type static diffusion cell set-up (Franz, 1975), with an exposed surface area of 0.64 cm^2 and a receptor compartment volume of 9.8 mL. Three Franz cells were run per sample type. The integrity of each skin sample was assessed by electrical resistance using the method and guidelines described by Davies *et al.* (Davies *et al.*, 2004) and only skin that produced an electrical resistance reading of > 10 kΩ was used. PBS solution (pH 7.4) was used as the receptor medium. An excess (infinite dose) of the active compound was applied via the donor compartment, as either a PBS solution (1 mL) or a gel formulation (200 mg). Sink conditions were maintained throughout the experiments. The skin was exposed to the formulation for 24 h in a water bath set to 37 °C. In-house stability studies, conducted previously, have demonstrated only a minimal reduction in ascorbic acid concentration (< 10 %) when prepared as an aqueous solution and left at 40 °C over a 24-hour time period. Therefore, it was felt that these Franz cell conditions were appropriate for this type of permeation assessment.

2.6 Post-Franz cell tissue preparation

Previous studies (Malm *et al.*, 2009; Van Nuffel *et al.*, 2016) have suggested that the optimum preparation method for ToF-SIMS analysis of cellular material is cryofixation followed by freeze-drying. However, for tissue samples, such as full thickness skin, it can be problematic to ensure that the freezing process is rapid enough to prevent the formation of ice crystals. It

has been suggested that without cryofixation, the high vacuum environment can compromise the integrity of the cellular structure (Malm *et al.*, 2009). However, it is anticipated that this would not significantly affect the localisation of, nor cause migration of, exogenous compounds within the skin. Following the Franz cell experiments described in Section 2.5, the excess formulation was removed from the skin with a spatula and the surface swabbed with a sponge that had been wet with distilled water, followed by a dry sponge. The skin samples were then dried flat under vacuum for 12 h before loading into the ToF-SIMS instrument for depth profile analysis. It is anticipated that as the skin is not being fixed, either chemically or through freezing, there may be some continued diffusion of the compounds through the skin tissue during this drying period. However, importantly, an identical preparation method is employed for all samples. Hence, any observed variation in permeation depth can be attributed to real differences between sample types.

2.7 ToF-SIMS analysis

Analysis was conducted using a ToF-SIMS IV instrument (IONTOF, GmbH) equipped with a single-stage reflectron analyser. Charge compensation of the sample was achieved using a low energy (< 20 eV) electron flood gun. For surface analysis, a Bi_3^+ beam energy of 25 keV, pulsed target current of ~ 0.3 pA and post-acceleration energy of 10 keV was employed. High current bunched (HC-BU) mode was used throughout and the primary ion dose density was maintained ≤ 1×10^{12} ions/cm^2 to ensure static conditions. The Bi_3^+ beam was directed at the sample at an angle of 45 °C with a focused size of 1-2 ≤ µm. Dual beam dynamic SIMS was conducted using an Ar gas cluster ion beam (GCIB) and Bi_3^+ liquid metal ion gun (LMIG) for sputtering and analysis respectively. The LMIG settings were as described previously. The GCIB used was a 20 keV Ar_{1900} beam with a target current of 10 nA. Non-interlaced depth profiling was employed, whereby the sputter and analysis ion beams do not operate simultaneously but at regular alternating intervals, with a single cycle consisting of 10 seconds sputtering, a 0.5 second pause and 3.25 seconds of analysis. A region of 500 µm × 500 µm was sputtered and a 200 µm × 200 µm region within the centre of the sputtered crater was analysed. Data was acquired and analysed using SurfaceLab 6 software (IONTOF, GmbH) and all exported peak intensities were dead time corrected and normalised to the total ion

count of their associated spectrum. Two areas were analysed per individual skin section, producing n=6 datasets for each sample type.

3. Results and Discussion

3.1 Validation of native tissue analysis using dynamic ToF-SIMS

Prior to permeation analysis, substantial method development was conducted to establish a reliable ToF-SIMS depth profiling method for native skin tissue. The final parameters provide a method that can profile through skin tissue at a practical rate and produce accurate chemical information. Optical profilometry was initially used to visualise the resulting sputter crater (Fig. S1) and stable ion intensities (Fig. 1a (i)) and consistent spatial distributions (Fig. 1b (i)) were observed as a function of depth for generic tissue markers, such as CN^-. Typical depth profile analysis relies on the calculation of a sputter rate, to provide a 3D chemical profile that is calibrated to sample depth. Preliminary analysis was conducted using optical profilometry to measure the depth of sputtered craters within the skin tissue. However, this method failed to produce repeatable results, confirming the difficulties previously shown to be associated with calculating a sputter rate for multi-layered samples (Bailey *et al.*, 2015; Seah *et al.*, 2015; Taylor *et al.*, 2015). The average sputter rate calculated from these experiments was 0.15 µm/s (data not shown) and this has been used to add an estimated skin depth scale to the ToF-SIMS profile graphs (Figures 1, 3 & 4) for reference purposes.

To circumvent the issues arising from sputter rate calculations, it was decided to instead look for inherent layer markers to identify the relative position within the skin when profiling. Although the *stratum corneum* contains different quantities of lipid species compared to the remaining epidermal layer, the nature of these lipids is not significantly different (Lampe *et al.*, 1983). Therefore, there were few ions that demonstrated obvious changes in intensity as a function of sample depth. However, one clearly observed difference was the absence of phospholipids within the *stratum corneum.* These lipids are present at high levels in the underlying epidermis but decrease towards the skin surface due to the keratinisation process (Lampe *et al.*, 1983). ToF-SIMS spectra for this lipid class are typically dominated by numerous phosphate-containing fragments, in particular, high intensity PO_3^-/PO_2^- ions

(Schaepe *et al.*, 2017; Sjövall *et al.*, 2014). These ions are absent from the initial portion of profiled skin tissue, which is clearly demonstrated when the data is presented as either a depth profile graph (Fig. 1a (ii)) or as a 3D ion map (Fig. 1b (ii)). The absence of these ions therefore indicates the location of the *stratum corneum*. As highlighted in Figure 1a (ii), at a specific depth into the profile (~ 170 sputter seconds) there is a significant increase in PO_3^- intensity, corresponding to the interface between the *stratum corneum* and underlying epidermis being reached. The use of ToF-SIMS to demonstrate this localisation of PO_3^- ions within skin has recently been shown by Sjövall *et al.* (Sjövall *et al*., 2014), through surface analysis of cross-sectioned porcine tissue. Using a similar sample set-up, we were able to corroborate this, highlighted in Figures 1c (i) and (ii), and the use of the dynamic SIMS approach allowed novel observation of this PO_3^- absence *in situ*. When attempting to monitor the permeation of an active compound, this sample-specific location of the *stratum corneum* interface can therefore be used to assess whether the compound has successfully passed into the underlying epidermis, which is often the primary target for localised compound delivery.

For any given skin sample, the intrinsic *stratum corneum* thickness will vary, highlighted in Figure 1c (ii). Therefore, for depth profile analysis, the epidermal interface will be reached at different depths within any given analysis region, which explains the gradual PO_3^- intensity increase observed in Figure 1a (ii). However, for the purpose of defining the interface depth, the sputter time relating to the onset of this intensity increase, highlighted in Fig. 1a (ii), is taken. It should be noted that for the experiments conducted within this study, the PO_3^- intensity profile was obtained from a blank skin sample taken from an adjacent part of the same porcine ear tissue. This ensured the intensity profile was not affected by phosphate ions originating from the formulation.

3.2 Permeation enhancing effects of a supramolecular hydrogel formulation

The two compounds chosen for permeation analysis, ascorbic acid and ascorbyl glucoside, both produce a high intensity, negative molecular ion that is distinct from any fragment ions found in native skin tissue, specifically $C_6H_7O_6^-$ (*m/z*=175) for ascorbic acid and $C_{12}H_{17}O_{11}^-$ (*m/z*=337) for ascorbyl glucoside (Fig. 2). It is worth noting that the reference spectrum for

ascorbyl glucoside (Fig. 2b) also contains a peak at *m/z*=175, produced by the instrument-induced fragmentation of this molecule into ascorbic acid. These compounds were applied to the skin tissue as both a PBS solution and a supramolecular hydrogel formulation. A comparison of the gelator reference spectrum with the reference spectra of both ascorbic acid and ascorbyl glucoside is shown in Fig. S2. This confirms that there are no peaks originating from the gelator material that overlap with the molecular ion peaks of the two compounds.

Figure 3 contains example data showing successful depth profile analysis of skin tissue applied with the four different formulations, displaying the intensity of the respective molecular ion as a function of skin depth. The full data set is displayed in Figure S3. The data is presented as both depth profile graphs and 3D secondary ion maps, with the latter elucidating the lateral and axial distribution of these compounds within the skin tissue. For both compounds, ascorbic acid (Fig. 3a) and ascorbyl glucoside (Fig. 3b), the data obtained confirms that permeation is greatly enhanced through application of the gel formulation compared to the PBS solution. The hydrogel formulation significantly increases the overall intensity of the molecular ions for both compounds, indicating an overall increased concentration permeating into the skin. In addition, both gel-applied compounds appear to maintain an intensity above the control level much deeper into the skin compared to the PBS solutions.

Both gel-applied compounds show a significant initial increase in intensity, rather than the general decrease associated with the PBS solutions. These peaks in intensity are likely to be associated with localised “pools” of each active compound. For the ascorbyl glucoside gel (Fig. 3b), the decrease in intensity is prolonged until the *stratum corneum* interface has been reached. Yet for the ascorbic acid gel (Fig. 3a), the maximum ion intensity is reached at a point substantially past this interface, demonstrating the ability of the gel to increase the amount of ascorbic acid available in the underlying epidermal layer. This compound localisation can also be observed in the example 3D ion map associated with the ascorbic acid gel formulation, as a visible band of increased intensity (Fig. 3a).

Although permeation enhancement of ascorbic acid has been previously attempted (Lee *et al*., 2017, 2003; Naveed *et al*., 2011) it is difficult to compare the success of this hydrogel formulation with other studies, as they typically use chromatography-based techniques to assess permeation. These studies will therefore often report a concentration or flux value for transdermal permeation, which is hard to relate to the ToF-SIMS intensity profiles. However, in the most recent study published on ascorbic acid enhancement, the concentration (µg/ cm2) of ascorbic acid retained in the epidermis after 24h was assessed, following the application of different liposome-based formulations (Maione-Silva *et al*., 2019). This study, by Maione-Silva *et al*., demonstrated that application via these liposome formulations resulted in between ~1.3 and 2 times more ascorbic acid present in the epidermal layer compared to the control. The ToF-SIMS profiles for ascorbic acid permeation obtained in this study (Fig. 3a), demonstrate that in the epidermal region (between ~20 and 80 µm), the level of ascorbic acid present following the hydrogel application is at least 2 times more than for the PBS application. Although this is a rough estimate and cannot be directly calculated, it does suggest that these hydrogels are within the same level of effectiveness for enhancing epidermal delivery of ascorbic acid.

The 3D ion maps displayed in Figure 3 indicate that in general a relatively consistent permeation depth was reached across the 200 µm × 200 µm analysis area. However, the permeation depth of ascorbic acid (Fig. 3a) appears to vary noticeably across this region following the application of the gel formulation. This may be the result of several possible factors, including a varied skin structure, non-uniformity in gel application onto the skin or even a non-uniformity in the distribution of the compound within the gel formulation itself. This demonstrates the variation in distribution that can occur following topical application of a compound and highlights the importance of being able to monitor this in order to understand the impact it has on the efficiency of the overall delivery system.

Apparent for both types of formulation, a significant variation in overall ion intensity is observed between the two compounds, ascorbic acid (Fig. 3a) and ascorbyl glucoside (Fig. 3b). However, this cannot solely be attributed to permeation of a higher concentration of

ascorbic acid. It is well established that ion intensities in ToF-SIMS can be affected by the ionisation ability of individual molecules and also by the sample environment, a phenomenon known as the matrix effect (Sodhi, 2004). In particular for organic samples, it has been demonstrated that the co-localisation of other species present in the sample can have a profound effect on ionisation and can result in both ion enhancement and suppression effects (Jones *et al*., 2007). Therefore, the effect of this on these samples needs to be further investigated. However, irrespective of overall ion intensity, there is a significant difference in permeation depth between the two compounds. This is apparent for both types of formulation and demonstrates that ascorbyl glucoside does not permeate as readily into the skin as ascorbic acid, most likely due to its increased molecular weight and hydrophilicity. It is, however, difficult to corroborate this data with previous results, as the permeation of vitamin c derivatives is a poorly researched area, highlighted in recent reviews by both Stamford and Pullar (references). There is only one study to date focused on the permeation of ascorbyl glucoside and currently no studies have made a comparative assessment of the permeation of ascorbyl glucoside and ascorbic acid.

3.3 Insights into the enzymatic breakdown of ascorbyl glucoside

Ascorbyl glucoside is employed in cosmetic products as an aqueous-stable derivative that is enzymatically converted into ascorbic acid within the skin. It was therefore proposed that the ToF-SIMS *in situ* analysis may be able to detect this enzymatic breakdown. Initial examination of the data showed that following application of ascorbyl glucoside as both the PBS solution and the gel formulation the molecular ion for ascorbic acid could also be detected within the skin tissue, as demonstrated by the example data shown in Figure 4. The full data set is displayed in Figure S4. However, as previously shown (Fig. 2b), the molecular ion peak for ascorbic acid (*m/z* = 175) is also present in the ToF-SIMS reference spectrum for ascorbyl glucoside, due to instrument-induced fragmentation. Therefore, for the samples applied with ascorbyl glucoside, it was first important to distinguish between the intensity of ascorbic acid related to instrument-induced fragmentation vs. the intensity produced through enzymatic conversion. To investigate this, the intensity ratio between the peaks at *m/z* = 337 (ascorbyl glucoside) and *m/z* = 175 (ascorbic acid) was calculated for a reference sample, consisting of

a homogenised mixture of skin tissue and ascorbyl glucoside, and then compared to the ratio observed throughout the depth profiles. On examination of the depth profile data, it was observed that this ratio varied from the reference, with a higher presence of ascorbic acid associated with increasing skin depth. This can therefore be attributed to the enzymatic conversion of ascorbyl glucoside, confirming that this transformation can be detected using the ToF-*SIMS in situ* analysis method.

It was also observed that this degradation of ascorbyl glucoside varied significantly depending on whether it had been applied to the skin in a PBS solution or as the gel formulation. In general, for the PBS treated samples (Fig. 4, blue data), the levels of ascorbic acid relative to ascorbyl glucoside rapidly increase with skin depth and both ions shown an early decrease in intensity, reaching a control level close to the stratum corneum interface. This demonstrates that, when delivered as a PBS solution, the permeation of ascorbyl glucoside is restricted to the stratum corneum and is undergoing rapid enzymatic breakdown into ascorbic acid before the underlying epidermis has been reached. In contrast, the gel formulation appears to show the opposite trend (Fig. 4, red data). The initial ratio between the two peak intensities is similar to the one observed for the reference sample and this is maintained until the stratum corneum has been passed. The intensities of the ascorbyl glucoside and ascorbic acid ions then start to simultaneously decrease and increase respectively. This suggests that not only is the gel formulation delivering more ascorbyl glucoside into the skin (Fig. 3), but that it is also preserving the degradation of this compound until after the stratum corneum has been passed. This results in a maximum ascorbic acid intensity in the underlying epidermal layer, the intended target for clinical efficacy. The 3D ion maps for both the ascorbyl glucoside ion (*m/z*=337), and ascorbic acid ion (*m/z*=175) also highlight this phenomenon (Fig. 4b). The ascorbyl glucoside ion displays a high intensity at the surface of the skin. Whereas, the intensity of the ascorbic acid ion is clearly reduced at the surface of the skin and the maximum intensity is located deeper within the skin tissue. Although this data is purely preliminary and the method could be further optimised, it highlights the huge potential of ToF-SIMS to assess the in situ conversion of vitamin c derivatives, something which has not yet been achieved.

4. Conclusions

This work has shown the successful incorporation of both ascorbic acid and ascorbyl glucoside into a supramolecular hydrogel formulation using an amphiphilic gemini imidazolium-based surfactant. It was demonstrated that this hydrogel formulation was able to significantly enhance the localised skin delivery of both compounds, resulting in an increased concentration residing in the epidermal layer of the skin, the intended delivery target for a cosmetic active ingredient. Significantly, it was shown that the hydrogel formulation was also able to preserve the enzymatic conversion of ascorbyl glucoside to ascorbic acid until after the *stratum corneum* barrier had been passed. This confirms the huge potential of these hydrogel formulations as topical delivery vehicles.

The observation of this enhanced localised skin permeation was achieved using an emerging technique in the field of skin permeation analysis, ToF-SIMS. Using this technique, we were able to successfully produce accurate 3D chemical profiles to monitor the permeation of these compounds through ex-vivo porcine skin. This enabled compound permeation and localisation to be studied in situ across the individual skin layers. This in situ analysis offers an alternative approach to explore compound localisation in the skin, without the need to overcome the previously discussed issues regarding layer isolation. These include method variability, extraction of specific compounds and tissue exposure to heat and exogenous chemicals. This work has also highlighted the advantage of monitoring the spatial distribution of compounds within skin tissue and the unique ability of ToF-SIMS to assess this both laterally and as a function of depth.

Although this method has been used here to monitor compound permeation through the skin, it has the potential to provide key information in a multitude of other biological applications. We have demonstrated the ability of this technique to chemically distinguish between subtly different tissue layers and also monitor compound degradation occurring within the tissue. This is applicable to both the intended degradation of derivatives/pro-drugs and the production of unwanted metabolites. It is therefore foreseeable that this method could be used to provide detailed analysis of exogenous compounds within other complex tissue matrixes, for example

analysis of ocular drug delivery or compound absorption within the gastrointestinal tract organs.

Acknowledgements

This work was supported by the following funding: The University of Nottingham Centre for Doctoral Training in Targeted Therapeutics and Formulation Science, Walgreens Boots Alliance and the EPSRC (grant EP/ I01375X/1); The Knowledge Transfer Partnership between The University of Nottingham, Walgreens Boots Alliance and Innovate UK (grant no. 510929); EU ERDF (FEDER) funds and the Spanish Government (grant TEC2017-85059-C3-2-R). All relevant data can be accessed through the Nottingham Research Data Management Repository at http://dx.doi.org/10.17639/nott.6171.

References

Alvarez-Román, R., Naik, A., Kalia, Y.N., Fessi, H., Guy, R.H., 2004a. Visualization of skin penetration using confocal laser scanning microscopy. Eur. J. Pharm. Biopharm. 58, 301–316. https://doi.org/https://doi.org/10.1016/j.ejpb.2004.03.027

Alvarez-Román, R., Naik, A., Kalia, Y.N., Guy, R.H., Fessi, H., 2004b. Skin penetration and distribution of polymeric nanoparticles. J. Control. Release 99, 53–62. https://doi.org/https://doi.org/10.1016/j.jconrel.2004.06.015

Ashtikar, M., Matthäus, C., Schmitt, M., Krafft, C., Fahr, A., Popp, J., 2013. Non-invasive depth profile imaging of the stratum corneum using confocal Raman microscopy: First insights into the method. Eur. J. Pharm. Sci. 50, 601–608. https://doi.org/https://doi.org/10.1016/j.ejps.2013.05.030

Bailey, J., Havelund, R., Shard, A.G., Gilmore, I.S., Alexander, M.R., Sharp, J.S., Scurr, D.J., 2015. 3D ToF-SIMS Imaging of Polymer Multilayer Films Using Argon Cluster Sputter Depth Profiling. ACS Appl. Mater. Interfaces 7, 2654–2659. https://doi.org/10.1021/am507663v

Barbero, A.M., Frasch, H.F., 2016. Effect of Frozen Human Epidermis Storage Duration and Cryoprotectant on Barrier Function using Two Model Compounds. Skin Pharmacol. Physiol. 29, 31–40. https://doi.org/10.1159/000441038

Bendich, A., Machlin, L.J., Scandurra, O., Burton, G.W., Wayner, D.D.M., 1986. The antioxidant role of vitamin C. Adv. Free Radic. Biol. Med. 2, 419–444. https://doi.org/https://doi.org/10.1016/S8755-9668(86)80021-7

Celebi, D., Guy, R.H., Edler, K.J., Scott, J.L., 2016. Ibuprofen delivery into and through the skin from novel oxidized cellulose-based gels and conventional topical formulations. Int. J. Pharm. 514, 238–243. https://doi.org/https://doi.org/10.1016/j.ijpharm.2016.09.028

Chughtai, K., Heeren, R.M.A., 2010. Mass Spectrometric Imaging for Biomedical Tissue Analysis. Chem. Rev. 110, 3237–3277. https://doi.org/10.1021/cr100012c

Darr, D., Combs, S., Dunston, S., Manning, T., Pinnell, S., 1992. Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage. Br. J. Dermatol. 127, 247–253. https://doi.org/10.1111/j.1365-2133.1992.tb00122.x

Davies, D.J., Ward, R.J., Heylings, J.R., 2004. Multi-species assessment of electrical resistance as a skin integrity marker for in vitro percutaneous absorption studies. Toxicol. Vitr. 18, 351–358. https://doi.org/https://doi.org/10.1016/j.tiv.2003.10.004

Farris, P.K., 2005. Topical Vitamin C: A Useful Agent for Treating Photoaging and Other Dermatologic Conditions. Dermatologic Surg. 31, 814–818. https://doi.org/10.1111/j.1524-4725.2005.31725

Franz, T.J., 1975. Percutaneous Absorption. On the Relevance of in Vitro Data. J. Invest. Dermatol. 64, 190–195. https://doi.org/https://doi.org/10.1111/1523-1747.ep12533356

Freudiger, C.W., Min, W., Saar, B.G., Lu, S., Holtom, G.R., He, C., Tsai, J.C., Kang, J.X., Xie, X.S., 2008. Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy. Science (80-.). 322, 1857 LP-1861.

Friend, D.R., 1992. In vitro skin permeation techniques. J. Control. Release 18, 235–248. https://doi.org/https://doi.org/10.1016/0168-3659(92)90169-R

Gerger, A., Koller, S., Weger, W., Richtig, E., Kerl, H., Samonigg, H., Krippl, P., Smolle, J., 2006. Sensitivity and specificity of confocal laser-scanning microscopy for in vivo diagnosis of malignant skin tumors. Cancer 107, 193–200. https://doi.org/10.1002/cncr.21910

Heber, G.K., Markovic, B., Hayes, A., 2006. An immunohistological study of anhydrous topical ascorbic acid compositions on ex vivo human skin. J. Cosmet. Dermatol. 5, 150–156. https://doi.org/10.1111/j.1473-2165.2006.00242.x

Hewitt, P.G., Perkins, J., Hotchkiss, S.A.M., 2000. Metabolism of Fluroxypyr, Fluroxypyr Methyl Ester, and the Herbicide Fluroxypyr Methylheptyl Ester. I: During Percutaneous Absorption through Fresh Rat and Human Skin In Vitro. Drug Metab. Dispos. 28, 748 LP-754.

Holmes, A.M., Scurr, D.J., Heylings, J.R., Wan, K.-W., Moss, G.P., 2017. Dendrimer pre-treatment enhances the skin permeation of chlorhexidine digluconate: Characterisation by in vitro percutaneous absorption studies and Time-of-Flight Secondary Ion Mass Spectrometry. Eur. J. Pharm. Sci. 104, 90–101. https://doi.org/https://doi.org/10.1016/j.ejps.2017.03.034

Humbert, P.G., Haftek, M., Creidi, P., Lapière, C., Nusgens, B., Richard, A., Schmitt, D., Rougier, A., Zahouani, H., 2003. Topical ascorbic acid on photoaged skin. Clinical, topographical and ultrastructural evaluation: double-blind study vs. placebo. Exp. Dermatol. 12, 237–244. https://doi.org/10.1034/j.1600-0625.2003.00008.x

Jacques-Jamin, C., Duplan, H., Rothe, H., Vaillant, O., Eilstein, J., Grégoire, S., Cubberley, R., Lange, D., Ellison, C., Klaric, M., Hewitt, N., Schepky, A., 2017. Comparison of the Skin Penetration of 3 Metabolically Stable Chemicals Using Fresh and Frozen Human Skin. Skin Pharmacol. Physiol. 30, 234–245.

Jones, E.A., Lockyer, N.P., Vickerman, J.C., 2007. Mass spectral analysis and imaging of tissue by ToF-SIMS—The role of buckminsterfullerene, C60+, primary ions. Int. J. Mass Spectrom. 260, 146–157. https://doi.org/https://doi.org/10.1016/j.ijms.2006.09.015

Judd, A.M., Scurr, D.J., Heylings, J.R., Wan, K.-W., Moss, G.P., 2013. Distribution and Visualisation of Chlorhexidine Within the Skin Using ToF-SIMS: A Potential Platform for the Design of More Efficacious Skin Antiseptic Formulations. Pharm. Res. 30, 1896–1905. https://doi.org/10.1007/s11095-013-1032-5

Kezutyte, T., Desbenoit, N., Brunelle, A., Briedis, V., 2013. Studying the penetration of fatty acids into human skin by ex vivo TOF-SIMS imaging. Biointerphases 8, 3. https://doi.org/10.1186/1559-4106-8-3

Kubo, A., Ishizaki, I., Kubo, A., Kawasaki, H., Nagao, K., Ohashi, Y., Amagai, M., 2013. The stratum corneum comprises three layers with distinct metal-ion barrier properties. Sci. Rep. 3, 1731. https://doi.org/10.1038/srep01731

Lampe, M.A., Williams, M.L., Elias, P.M., 1983. Human epidermal lipids: characterization and modulations during differentiation. J. Lipid Res. 24, 131–140.

Lapteva, M., Mondon, K., Möller, M., Gurny, R., Kalia, Y.N., 2014. Polymeric Micelle Nanocarriers for the Cutaneous Delivery of Tacrolimus: A Targeted Approach for the Treatment of Psoriasis. Mol. Pharm. 11, 2989–3001. https://doi.org/10.1021/mp400639e

Limón, D., Amirthalingam, E., Rodrigues, M., Halbaut, L., Andrade, B., Garduño-Ramírez, M.L., Amabilino, D.B., Pérez-García, L., Calpena, A.C., 2015. Novel nanostructured supramolecular hydrogels for the topical delivery of anionic drugs. Eur. J. Pharm. Biopharm. 96, 421–436. https://doi.org/https://doi.org/10.1016/j.ejpb.2015.09.007

Limón, D., Jiménez-Newman, C., Calpena, A.C., González-Campo, A., Amabilino, D.B., Pérez-García, L., 2017. Microscale coiling in bis-imidazolium supramolecular hydrogel fibres induced by the release of a cationic serine protease inhibitor. Chem. Commun. 53, 4509–4512. https://doi.org/10.1039/C6CC09392B

Limón, D., Jiménez-Newman, C., Rodrigues, M., González-Campo, A., Amabilino, D.B., Calpena, A.C., Pérez-García, L., 2017. Cationic Supramolecular Hydrogels for Overcoming the Skin Barrier in Drug Delivery. ChemistryOpen 6, 585–598. https://doi.org/10.1002/open.201700040

Malm, J., Giannaras, D., Riehle, M.O., Gadegaard, N., Sjövall, P., 2009. Fixation and Drying Protocols for the Preparation of Cell Samples for Time-of-Flight Secondary Ion Mass Spectrometry Analysis. Anal. Chem. 81, 7197–7205. https://doi.org/10.1021/ac900636v

Monteiro-Riviere, N.A., Wiench, K., Landsiedel, R., Schulte, S., Inman, A.O., Riviere, J.E., 2011. Safety Evaluation of Sunscreen Formulations Containing Titanium Dioxide and Zinc Oxide Nanoparticles in UVB Sunburned Skin: An In Vitro and In Vivo Study. Toxicol. Sci. 123, 264–280.

Murad, S., Tajima, S., Johnson, G.R., Sivarajah, A., Pinnell, S.R., 1983. Collagen Synthesis in Cultured Human Skin Fibroblasts: Effect of Ascorbic Acid and Its Analogs. J. Invest. Dermatol. 81, 158–162. https://doi.org/https://doi.org/10.1111/1523-1747.ep12543573

Nicoli, S., Santi, P., 2007. Suitability of Excised Rabbit Ear Skin—Fresh and Frozen—for Evaluating Transdermal Permeation of Estradiol. Drug Deliv. 14, 195–199. https://doi.org/10.1080/10717540601036849

Offord, E.A., Gautier, J.-C., Avanti, O., Scaletta, C., Runge, F., Krämer, K., Applegate, L.A., 2002. Photoprotective potential of lycopene, β-carotene, vitamin E, vitamin C and carnosic acid in UVA-irradiated human skin fibroblasts. Free Radic. Biol. Med. 32, 1293–1303. https://doi.org/https://doi.org/10.1016/S0891-5849(02)00831-6

Per, M., Thomas, G., B., E.M., Lina, H., 2017. Imaging mass spectrometry for novel insights into contact allergy – a proof-of-concept study on nickel. Contact Dermatitis 78, 109–116. https://doi.org/10.1111/cod.12911

Phillips, C.L., Combs, S.B., Pinnell, S.R., 1994. Effects of Ascorbic Acid on Proliferation and Collagen Synthesis in Relation to the Donor Age of Human Dermal Fibroblasts. J. Invest. Dermatol. 103, 228–232. https://doi.org/https://doi.org/10.1111/1523-1747.ep12393187

Pullar, J.M., Carr, A.C., Vissers, M.C.M., 2017. The Roles of Vitamin C in Skin Health. Nutrients 9, 866. https://doi.org/10.3390/nu9080866

Pyatski, Y., Zhang, Q., Mendelsohn, R., Flach, C.R., 2016. Effects of permeation enhancers on flufenamic acid delivery in Ex vivo human skin by confocal Raman microscopy. Int. J. Pharm. 505, 319–328. https://doi.org/https://doi.org/10.1016/j.ijpharm.2016.04.011

Rajadhyaksha, M., Grossman, M., Esterowitz, D., Webb, R.H., Rox Anderson, R., 1995. In Vivo Confocal Scanning Laser Microscopy of Human Skin: Melanin Provides Strong Contrast. J. Invest. Dermatol. 104, 946–952. https://doi.org/https://doi.org/10.1111/1523-1747.ep12606215

Rodrigues, M., Calpena, A.C., Amabilino, D.B., Garduño-Ramirez, M.L., Pérez-García, L., 2014. Supramolecular gels based on a gemini imidazolium amphiphile as molecular material for drug delivery. J. Mater. Chem. B 2, 5419–5429. https://doi.org/10.1039/C4TB00450G

Saar, B.G., Contreras-Rojas, L.R., Xie, X.S., Guy, R.H., 2011. Imaging Drug Delivery to Skin with Stimulated Raman Scattering Microscopy. Mol. Pharm. 8, 969–975. https://doi.org/10.1021/mp200122w

Schaepe, K., Werner, J., Glenske, K., Bartges, T., Henss, A., Rohnke, M., Wenisch, S., Janek, J., 2017. ToF-SIMS study of differentiation of human bone-derived stromal cells: new insights into osteoporosis. Anal. Bioanal. Chem. 409, 4425–4435. https://doi.org/10.1007/s00216-017-0386-7

Seah, M.P., Havelund, R., Shard, A.G., Gilmore, I.S., 2015. Sputtering Yields for Mixtures of Organic Materials Using Argon Gas Cluster Ions. J. Phys. Chem. B 119, 13433–13439. https://doi.org/10.1021/acs.jpcb.5b06713

Sjövall, P., Greve, T.M., Clausen, S.K., Moller, K., Eirefelt, S., Johansson, B., Nielsen, K.T., 2014. Imaging of Distribution of Topically Applied Drug Molecules in Mouse Skin by Combination of Time-of-Flight Secondary Ion Mass Spectrometry and Scanning Electron Microscopy. Anal. Chem. 86, 3443–3452. https://doi.org/10.1021/ac403924w

Sodhi, R.N.S., 2004. Time-of-flight secondary ion mass spectrometry (TOF-SIMS):-versatility in chemical and imaging surface analysis. Analyst 129, 483–487. https://doi.org/10.1039/B402607C

Stamford, N.P.J., 2012. Stability, transdermal penetration, and cutaneous effects of ascorbic acid and its derivatives. J. Cosmet. Dermatol. 11, 310–317. https://doi.org/10.1111/jocd.12006

Starr, N.J., Johnson, D.J., Wibawa, J., Marlow, I., Bell, M., Barrett, D.A., Scurr, D.J., 2016. Age-Related Changes to Human Stratum Corneum Lipids Detected Using Time-of-Flight Secondary Ion Mass Spectrometry Following in Vivo Sampling. Anal. Chem. 88, 4400–4408. https://doi.org/10.1021/acs.analchem.5b04872

Taylor, A.J., Graham, D.J., Castner, D.G., 2015. Reconstructing accurate ToF-SIMS depth profiles for organic materials with differential sputter rates. Analyst 140, 6005–6014. https://doi.org/10.1039/C5AN00860C

Van Nuffel, S., Parmenter, C., Scurr, D.J., Russell, N.A., Zelzer, M., 2016. Multivariate analysis of 3D ToF-SIMS images: method validation and application to cultured neuronal networks. Analyst 141, 90–95. https://doi.org/10.1039/C5AN01743B

Zhou, W., Liu, W., Zou, L., Liu, W., Liu, C., Liang, R., Chen, J., 2014. Storage stability and skin

permeation of vitamin C liposomes improved by pectin coating. Colloids Surfaces B Biointerfaces 117, 330–337. https://doi.org/https://doi.org/10.1016/j.colsurfb.2014.02.036