Dendrimer-mediated permeation enhancement of chlorhexidine digluconate: determination of *in vitro* skin permeability and visualisation of dermal distribution

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**Abbreviations**

CHX - Chlorhexidine

CHG – Chlorhexidine digluconate

HEC – hydroxyethyl cellulose

LoD/LoQ – Limit of detection/limit of quantification

PAMAM – Poly(amidoamine)

SC – *Stratum corneum*

ToF-SIMS – Time-of-Flight Secondary Ion Mass Spectrometry

**Abstract**

Chlorhexidine digluconate (CHG) is a cationic bisbiguanide used as the first-line skin antiseptic prior to surgery in the UK due to its favourable efficacy and safety profile, high affinity for skin binding and minimal reports of resistance. Despite this, bacteria remain within deeper skin layers, furrows and appendages that are considered inaccessible to CHG, due to its poor dermal penetration. In this study, a third generation, polyamidoamine dendrimer (G3 PAMAM-NH2) was utilised to improve dermal penetration of CHG. A topical gel formulation was optimised to maximise CHG delivery (containing 0.5% gelling agent and 4% drug), followed by drug and dendrimer co-formulation into a commercially viable gel. The gel containing 4% CHG and 1mM PAMAM dendrimer significantly increased the depth permeation of CHG compared to the commercial benchmark (Hibiscrub®, containing 4% w/v CHG) (p<0.05). The optimised formulation was further characterised using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS), which indicated that the depth of dermal penetration achieved was sufficient to reach the skin strata that typically harbours pathogenic bacteria, which is currently inaccessible by commercial CHG formulations. This study therefore indicates that a G3 PAMAM-NH2 dendrimer gel may be viable strategy as a permeation enhancer of CHG, for improved skin antisepsis in those at risk of a skin or soft tissue infection as a result of surgical intervention.

**1.0 Introduction**

Chlorhexidine digluconate (CHG) is a cationic bisbiguanide which is typically used for skin antisepsis prior to surgery. The National Institute for Health and Care Excellence (NICE) guidelines for prevention and treatment of surgical site infections name an aqueous or alcohol based solution of chlorhexidine (CHX) as the first choice antiseptic in the UK1. This status may be attributed to its ability to effectively kill surface bacteria with minimal reports of resistance, low mammalian toxicity and a high affinity for skin surface binding2. It is preferred over other antiseptics such as povidone iodine as it retains its antimicrobial effects when in contact with blood3, and CHG bathing has been shown to reduce acquired infection rates of methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE)4, bacteria which may contribute to a predicted, imminent antimicrobial crisis5.

Bacteria are understood to reside not only on the skin surface, but deep within the epidermis and they have also been found to consistently reside within the dermis6,7. Nakatsuji *et al.* detected DNA encoding for bacterial 16S rRNA genes below the maximal depth of follicles in facial skin (3.0 mm), and below the eccrine glands in palm skin (1.5 mm). PCR amplification, Gram staining, immunostaining and *in situ* hybridisation was performed to identify the bacterial class and phylum. Bacteria were understood to be diverse, with high proportions of Proteobacteria present across all samples. *S. epidermidis* was routinely detected below the epidermal basement membrane, and *Pseudomonas spp.* was readily detected in the dermis outside of appendageal structures7.

In addition to subepidermal bacteria, bacteria are understood to also reside in skin folds and appendages8. This flora (inaccessible by traditional skin sampling methods) is estimated to be present at a depth of 400-700 μm9, the approximate depth of hair follicles. The flora that resides subepidermally harbours the opportunity to become pathogenic if the skin barrier is reduced or removed, for example, at the site of a surgical incision. Despite the widespread, long term use of CHG in the healthcare setting, its skin permeability is poor10,11 due to its high molecular weight (897.8 g/mol12), high aqueous solubility (50 mg/mL13), low log P (0.013314) and ionisation state at physiological pH2,15*.* Therefore, the ability of CHG to target potentially pathogenic bacteria in deeper skin layers is diminished.

There is, therefore, an impetus to improve the efficacy of existing antimicrobials because of a predicted, imminent antimicrobial crisis5. A lack of return for investments in antimicrobial research and development prevents pharmaceutical companies from assigning resources to antimicrobial drug discovery, preferring to place resources elsewhere for a higher chance of a safe investment return16,17. Improving the efficacy of existing antimicrobials, such as CHG, may aid in the prevention of antimicrobial infections, without the vast cost associated with novel drug developments and the risk of premature antimicrobial resistance.

For quantification of transdermal drug permeation *in vitro*, the well-established OECD guidelines18,19 recommend the use of diffusion cells. In addition to this method, tape stripping is a robust and simple technique that allows drug permeation within the upper skin layers to be determined, which is particularly useful when intradermal penetration requires investigation. Thus, diffusion cell studies with associated tape stripping techniques are two well-established methods of quantifying drug permeation into and across skin. Despite this, it cannot provide information regarding drug distribution across the skin. Such information may be useful to determine whether a drug permeates *via* specific pathways (e.g., through appendages), or to determine the specific depth of drug permeation.

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) is an analytical technique which can simultaneously provide a mass spectra of the sample and images of a high spatial resolution (<1 μm20). In addition, ToF-SIMS removes the need to pool tape strips for HPLC detection when skin permeation is poor in deeper skin layers21. In addition, the images produced from ToF-SIMS allow visualisation of the homogeneity of drug distribution across tape stripped skin, which compliments the quantitative results obtained from traditional HPLC sample analysis. This imaging technique has previously been used to view native skin components22,23 in both healthy24–26 and diseased27–29 tissue . Drug deposition imaging within the skin is a relatively new yet developing application of ToF-SIMS30–32.

Poly(amidoamine) (PAMAM) dendrimers are comprised of multiple branching arms deriving from a central diamine core33. They exhibit numerous properties that make them desirable for use as percutaneous penetration enhancers, such as their uniform molecular weight, their predictable, reproducible 3D architecture34, their biocompatibility and their water solubility35. Furthermore, dendrimers are able to accommodate small molecules in their core or complex drugs to their surface for modified physicochemical properties, which may serve to enhance drug solubility and stability36–39. Thus, this study aimed to create a co-formulation of CHG and a G3 PAMAM-NH2 for simple and convenient skin application, and to enhance the permeation of CHG into deeper skin layers to a greater degree than that currently achievable by Hibiscrub® (4% w/v CHG), the chosen commercial benchmark. In this work, it was demonstrated through Franz-type diffusion cell studies analysed using both HPLC and ToF-SIMS that a G3 PAMAM-NH2 dendrimer (1mM concentration) significantly enhanced the permeation of CHG (4% w/v) into porcine skin when compared to the commercial benchmark, Hibiscrub® (contains 4% w/v CHG) (p<0.05). CHG permeation was uniform across the skin without apparent permeation *via* specific channels, such as appendages, and the mechanism of such permeation enhancement appears to be due to occlusive effects as opposed to specific drug-dendrimer interactions.

**2.0 Experimental Section**

**2.1 Materials**

G3 PAMAM-NH2 dendrimer (20% w/v in methanol), 2-hydroxyethyl cellulose (HEC, molecular weight ~1300000), trimethylamine (25% w/v in water), HPLC grade methanol (> 99.8%,), glacial acetic acid (> 99/7%) and HPLC grade acetonitrile (> 99.8%) were obtained from Sigma Aldrich. Chlorhexidine digluconate (CHG, 20% w/v in water) and sodium octane-1-sulfonate monohydrate (99+% crystalline) were obtained from Alfa Aesar. Ethanol absolute was obtained from VWR. Glycerol was purchased from Acros Organics.

Franz-type diffusion cells were obtained from Soham Scientific (Cambridge, UK). Syringe filters (0.2 μm, 15 mm diameter), HPLC *via*ls (1.5 mL crimp neck *via*l, 32 × 11.6 mm) and crimper caps (1.0mm), D-squame™ tape strips (standard) were obtained from Fisher Scientific. Loctite® super glue was obtained from Lyreco (Shropshire, UK).

**2.2 Methods**

**2.3 Co-formulation of CHG and G3 PAMAM-NH2 dendrimer**

Prior to drug-dendrimer co-formulation, delivery of CHG from the gel formulation was optimised through the formulation of a series of gels. An ethanol-based gel was chosen as they are fast acting, economical and convenient to use 8,40,41. In addition, gels apply to the skin smoothly, are aesthetically pleasing, and the ethanol smell is associated with cleanliness. Gels have also been shown to be preferred over other conventional topical formulations, such as creams42 and ointments43.

Gels were formulated by firstly mixing all wet ingredients (water, ethanol, CHG, glycerol), then by slow addition of HEC. For the formulations containing PAMAM dendrimer, CHG and PAMAM were separately stabilised in the ethanol:water mixture prior to addition of glycerol and HEC. Formulations were allowed to set for 24 h at room temperature before *in vitro* experiments were conducted.

Prior to drug-dendrimer co-formulation, a CHG containing gel was firstly optimised by altering the concentration of gelling agent (HEC; 0.5-4% w/w) and CHG (1-4%) concentration. The selected parameter was altered, *in vitro* percutaneous absorption studies were conducted, and the formulation that delivered the most CHG, as indicated by tape strip analysis using HPLC, was carried forward to the next stage of optimisation. Finally, the concentration of PAMAM dendrimer was altered (0.5 mM-1 mM) and the formulation that delivered the most CHG in vitro was taken forward for analysis by ToF-SIMS, alongside the commercial benchmark and the optimised gel formulation without the PAMAM dendrimer.

**2.4 Porcine skin preparation**

Porcine skin sourcing and preparation follows guidance from OECD for *in vitro* percutaneous absorption18, though full thickness rather than split thickness porcine skin was used. Transepidermal water loss (TEWL) was measured for each skin sample to check its integrity using a Biox Aquaflux™ meter (model AF200). The TEWL probe was allowed to equilibrate for 2 min before any TEWL measurements of skin samples were taken. Any skin sample with a TEWL measurement 4 × above or below the mean TEWL measurement was discarded44. Franz-type diffusion cells were also prepared by following the methods of Judd *et al*45, with the addition of a thin layer of Loctite® superglue around the outer rim of both the receptor and donor chamber to reduce phosphate buffered saline or applied formulation from leaking. Cells were placed in a water bath set to 37 oC. The absence of individually water-jacketed Franz cells meant that, with a water level no higher than the top of the receiver compartment, a skin surface temperature of 32 ± 1 oC could be maintained. This setup was allowed to equilibrate for 30 m, after which 1 mL of formulation was added to each donor chamber.

**2.5 Franz-type diffusion cell studies with associated tape stripping for HPLC and ToF-SIMS analysis**

At the end of the 24 h experiment, Franz- type diffusion cells were disassembled and excess formulation remaining on the skin surface was removed using an absorbent paper towel. Skin was allowed to air dry at ambient temperature, after which 21 consecutive D-squame™ tape strips were firmly pressed onto the treated area of skin using a roller and quickly removed from each skin sample to remove *stratum corneum* (SC) corneocytes. The first three tape strips were retained separately and the remaining strips were pooled into groups: 4-6, 7-10, 11-16 and 17-21. Tape strips were weighed before and after stripping to allow for gravimetric analysis of tape strip weights and normalisation of CHG content as described previously46,47. CHG was extracted from all tape strips in 5 mL of mobile phase. Tape stripping studies for ToF-SIMS analysis also used the method described above, however tape strips for ToF-SIMS analysis were placed sticky-side up onto glass microscope slides following application to the skin, and secured in place with double-sided Sellotape™.

HPLC analysis of both CHG receptor phase and tape strip samples used the method detailed previously31. A Thermo Scientific guard column with replaceable guard cartridges (Thermo Scientific, C18 10 mm, 5 μm) were used to ensure HPLC pressure remained stable throughout the *in vitro* study. The limit of detection (LoD) and limit of quantification (LoQ) were calculated from this calibration graph according to the following equations:

**(Equation 1)**

**(Equation 2)**

The experimental mobile phase was used as the solvent for all CHG extractions and was validated on the Shimadzu system (R2 value 0.9992). The LoD was calculated to be 0.362 µg/mL and the LoQ was calculated to be 1.098 µg/mL.

**2.6 Cryosectioning study for ToF-SIMS analysis**

Skin samples were disassembled from the Franz-type diffusion cell apparatus following the 24 h diffusion cell study. Samples were immediately placed on an aluminium block and fresh frozen with liquid nitrogen. Upon freezing, the samples were cryosectioned using a Leica CM3050 Research Cryostat, obtaining sections of 20 µm thickness, using a “partial OCT embedding cryomicrotomy” method pioneered by Kubo *et al.* 48. The skin slices were then thaw mounted on a glass slide and stored at -20 oC prior to ToF-SIMS analysis.

**2.7 ToF-SIMS Analysis**

ToF-SIMS analysis was performed using a ToF-SIMS IV instrument (IONTOF, GmbH) with a Bi3+ cluster source. A primary ion energy of 25 KeV was used; the primary ion dose was preserved below 1 × 1012 per cm2 to ensure static conditions. Pulsed target current of approximately 0.3 pA, and post-acceleration energy of 10 keV were employed throughout the sample analysis. The mass resolution for the instrument was 7000 at *m/z* 28. The scanned area of the tape strips samples was 4 mm × 4 mm, encompassing the skin area exposed to the formulation during Franz-type diffusion cell experiments. The raster size for individual tiles within the 4 mm × 4 mm tape strip analysis area was 500 × 500 µm. The analysis was carried out using 1 shot/pixel with a total of one scan. An analysis area of 1.5 mm × 3 mm was employed for the skin cross-sections. The raster size for individual tiles within the 1.5 mm × 3 mm skin cross-section analysis area was 400 × 400 µm. The analysis was carried out using 1 shot/pixel with a total of three scans. Both sample types were analysed at a pixel density of 100 pixels/mm. An ion representing biological material and therefore indicative of skin (skin marker) was identified as CN- and was used to threshold the data sets from tape strips. Thresholding in this instance refers to the process of using the ToF-SIMS ion image to create a region of interest. A specific ion representing the tissue, in this case CN-, is selected to discriminate between tissue and adhesive by creating a region of interest. The remaining area is then excluded from the analysis in order to exclude an inclusion of non-tissue material, in this case the adhesive present in the fissures of the tape strips. This method also allows the amount of tissue to be accounted for when the data is subsequently normalised to total ion intensity of these tissue specific regions of interest. This method of using ToF-SIMS to analyse tape-strips have been demonstrated previously by several researchers26,49,50. CN- is a common fragment observed in organic materials such as biological specimens. Therefore, this secondary ion was used to track the presence of corneocytes extracted on the tape strips. The data was reconstructed to remove the data from the adhesive tape material found between the fissures in the stripped skin and therefore the data was only analysed from the skin material. Following this, each image of the individual tape strip (4 mm × 4 mm) was divided into four smaller data sets of 2 mm × 2 mm, which results in four repeats (*n = 4*) for each sample and their intensities were normalised to the total ion intensity. All ToF-SIMS data was acquired in the negative mode based on previous work31 as the diagnostic ions of interest specific to CHG (namely C7H4N2Cl-) provided the greatest signal to noise ratio than the positive mode for the ions of interest specific to CHG. The use of this diagnostic ions in the negative mode provided a greater contrast for subsequent analysis of drug distribution within the tissue sample.

For measuring the depth of permeation of CHG from CHG treated cross sections, the line scan function and measurement editor tool in Surface Lab 7 software was utilised (**Figure S1)**. This tool allows the depth permeation of a chosen ion to be measured manually across a cross section (μm). The C7H4N2Cl- ion was used as a marker of the drug and was utilised to manually measure the depth permeation of the ion. Measurements were taken systematically across the entire width of each cross section sample for each formulation to account for differing epidermal thicknesses across the skin cross sections and the numerous wrinkles and furrows in the skin. Example of the line scan function and measurement editor tool used to estimate the permeation depth of CHG into the skin is illustrated in the supporting information **Figure S1.**

**2.8 Statistical Analysis of HPLC and ToF-SIMS data**

All data is displayed as the mean ± standard error of mean (SEM), followed by the sample number, *n*. All statistical analysis was undertaken using IBM SPSS. Data was first assessed for normality and homogeneity of variance using the Shapiro-Wilk and Levenes test respectively. If results indicated that the data was parametric, a One-Way ANOVA with Tukey’s post hoc test was conducted. If results indicated that the data was non-parametric, a Kruskal Wallis ANOVA with Dunn’s post hoc test was conducted.

**3.0 Results and Discussion**

**3.1 Formulation Optimisation and the effect of CHG-PAMAM Dendrimer Co-formulation on the *In Vitro* Permeation of CHG – Tape Stripping HPLC Analysis**

HPLC analysis of pooled tape strips following Franz-type diffusion cell experiments was performed in order to quantify the amount of CHG that has permeated into the SC of porcine skin tissue. The concentration of CHG per mg of SC material (µg/mg) recovered from pooled tape strips from each formulation following the 24 h Franz-type diffusion cell study is shown in **Figure 1**. Delivery of CHG from the gel formulation was optimised by altering the concentration of gelling agent, HEC (**Figure 1A**) and drug, CHG (**Figure 1B**) in the formulation. The optimised formulation (containing 0.5 % w/w HEC and 4% w/v CHG) was carried forward and formulated with either 0.5 mM or 1 mM G3 PAMAM-NH2 dendrimer and compared to the optimised gel formulation in the absence of the PAMAM dendrimer, and the commercial benchmark (**Figure 1C**).

The receptor fluid samples provided little information on CHG permeation as most samples analysed detected no CHG, and where CHG was detected, all samples were below the LoD (data not shown).

***Figure 1.*** *Results of tape stripping studies following Franz-type* in-vitro *diffusion cell experiments (presented as mean ± SEM) of CHG per mg of SC material weighed gravimetrically.* ***(a)*** *concentration of CHG detected from formulations (all containing 4% w/v CHG) altering the concentration of gelling agent (n ≥ 4),* ***(b)*** *concentration of CHG detected from formulations altering the concentration of drug (n ≥ 4),* ***(c)*** *concentration of CHG detected from formulations (all containing 0.5% w/v HEC) altering the concentration of PAMAM dendrimer and compared to the optimised formulation without dendrimer, and the commercial benchmark (µg/mg, n ≥ 4 for Hibiscrub® 4% w/v, 4% w/v CHG, 4% CHG-0.5mM PAMAM and 4% CHG-1mM PAMAM respectively). All pairwise comparisons indicate that a statistically significant result occurred (p <0.05). Number of repeats is indicated as “≥”, as removal of 21 tape strips from an individual sample was not always possible, due to the increased presence of interstitial fluid on latter tape strips, which in some instances caused the epidermis to tear, voiding the sample. This observation is consistent with a previous study*31*. Abbreviation: HEC, hydroxyethyl cellulose; CHG, chlorhexidine gluconate, PAMAM, polyamidoamine dendrimer.*

**Figure 1(A)** and **1(B)** indicates that decreasing the concentration of HEC and increasing the concentration of CHG in the formulation increases the permeation of CHG *in vitro*. An increase in viscosity reduces free movement of drug particles within the formulation, reducing the incidence in which a drug particle is able to come into contact with the skin surface, interact with it, and diffuse through the barrier51. Furthermore, increasing the concentration of drug applied to the skin surface increases the spontaneous movement of particles from an area of high concentration (the skin surface) to an area of low concentration (deeper skin layers), in accordance with Fick’s law52.

**Figure 1(C)** indicates that the addition of a G3 PAMAM-NH2 dendrimer to the formulation containing 4% w/v CHG increased the deposition of CHG within porcine skin compared to both the optimised gel without the addition of the PAMAM dendrimer, and the commercial benchmark. The 4% CHG-1mM PAMAM gel delivered significantly more CHG onto each tape strip than Hibiscrub® 4% w/v (p <0.05). This increase in CHG deposition from the co-formulation is particularly apparent from the upper tape strips of the 4% CHG-1mM PAMAM formulation. For example, there was a 2.7-fold increase in CHG permeation when comparing 4% CHG and 4% CHG-1mM PAMAM, which decreases to a 1.8-fold difference on tape strips 17-21 when comparing the same groups. This trend of reduced permeation enhancement effect with increasing tape strip number is consistent with previous studies that investigated CHG permeation enhancement31,53.

It was observed that after the 24 h Franz-type diffusion cell experiment, a viscous layer of formulation was visible on the skin surface (4% w/v CHG; 4% CHG-0.5mM PAMAM and 4% CHG-1mM PAMAM formulations). This increase in formulation viscosity was more pronounced in the formulations containing the PAMAM dendrimer, evidenced by the formulations ability to retain its shape after the donor compartment of the Franz-type diffusion cell was removed upon cell disassembly after 24 h. The presence of this viscous layer may cause an occlusive effect that disrupts the lipid arrangement in the SC thereby leading to a large increase in CHG permeation depth. This observation suggests that the PAMAM dendrimer has a physical effect on the skin barrier, independent of any potential drug-dendrimer interactions. Occlusion is a known mechanism of drug permeation enhancement, and occurs through skin lipid barrier disruption, increasing the intra-lipid space, through which drugs can permeate54,55. Furthermore, dendrimers have been shown to be hygroscopic56, further supporting the proposed mechanism of occlusion-enhanced skin permeation.

It was also found that the gel formulation containing 4% w/v CHG without dendrimer was able to deliver more CHG into the SC than Hibiscrub® (4% w/v CHG), when applied to the skin for 24 h (although this increase was only considered statistically significant on tape strips 4-6 and when comparing total CHG permeation across all tape strips). This result indicates that development of the vehicle improved the ability of CHG to permeate into porcine skin, independent of formulating the drug with a G3 PAMAM-NH2 dendrimer. The ability of simple vehicles to influence the permeation of drugs into and through skin has been discussed extensively in previous studies57–62, however it is often difficult to determine what effects are likely to occur from mixtures of several vehicles63–65 where covariance and synergy should be considered. The ability of the formulation to enhance CHG deposition compared to Hibiscrub® may be attributed to film formation effects66,67, resulting in increased residency time of the drug on the skin, drug saturation following ethanol evaporation68,69, and possibly occlusive31,45,56 and surface tension70–72 effects.

Nonetheless, the addition of 1mM G3 PAMAM-NH2 dendrimer significantly enhanced CHG permeation into porcine skin when compared to Hibiscrub® 4% w/v, indicating that the structure is able to act as a permeation enhancer to CHG, and that a simple, convenient co-formulation could be made with the ability to enhance CHG permeation for enhanced antisepsis effects. Furthermore, the study illustrates the ability of the dendrimer to act as a permeation enhancer specifically for hydrophilic drugs such as CHG. PAMAM dendrimer mediated percutaneous permeation enhancement has previously focused on poorly water soluble drugs45,71,72, as drug entrapment in the dendritic architecture is proposed to improve aqueous solubility and thus drug available for permeation in an applied aqueous vehicle73. This mechanism seems unlikely for PAMAM mediated enhancement of CHG, as encapsulation efficiency is increased when the drug and dendrimer interior are ionised, and when the molecular weight of the drug is within a 200-400 g/moL range; neither of which applies to this system74,75. Thus, further investigation is required to fully understand PAMAM dendrimer’s ability to enhance permeation of hydrophilic drugs.

**3.2 The Effect of CHG-PAMAM Dendrimer Co-formulation on the *In Vitro* Permeation of CHG – Tape Stripping ToF-SIMS Analysis**

Although HPLC analysis of pooled tape strips provided quantitative results regarding CHG permeation trends from respective formulations into porcine skin, the method does not confer any information detailing CHG spatial distribution within individual layers of skin. Therefore, an additional analytical technique was employed to gauge the spatial information of drug distribution across respective corneocyte layers. Additionally, ToF-SIMS requires a minimal amount of sample for sufficient analyte detection, therefore the micron sized range sample used in this study (4 mm × 4 mm tape strip area) was sufficient to provide spatial information representing the entirety of the treatment area. The permeation of CHG across the skin was tracked using the fragment ion C7H4N2Cl- as reported by Judd *et al.*45. This was confirmed by analysing untreated tape strips and those treated with the optimised 4% CHG formulation. The mass spectra produced by ToF-SIMS and the associated ion intensity images indicated a clear specificity of C7H4N2Cl- ion when the 4% CHG gel was applied, which was not present in untreated skin (**Figure S2**).

Secondary ion images of the analysed tape stripped area, which represents the exposed area of skin to CHG containing formulations, can be found in **Figure 2**. The formulations of interest were dictated by the results of the *in vitro* Franz-type diffusion cell experiment (**Figure 1**) and were; the optimised formulation without the addition of the PAMAM dendrimer (4% CHG); the optimised formulation with the PAMAM dendrimer at a concentration which was found to deliver the greatest concentration of CHG according to the *in vitro* experiment (4% CHG-1mM PAMAM); and the commercial benchmark (Hibiscrub®, containing 4% w/v CHG).

***Figure 2.*** *CHG ion C7H4N2Cl- ion intensity across untreated skin, Hibiscrub® 4% w/v, 4% w/v CHG optimised gel formulation and 4% CHG-1 mM PAMAM optimised gel formulation. Each image represents a 4 mm × 4 mm area of porcine skin. Abbreviation: CHG, chlorhexidine gluconate, PAMAM, polyamidoamine dendrimer. The intensity scales of all images are equal, allowing for direct comparison of the CHG signal intensities between the samples.*

The distribution of CHG across the 4 mm × 4 mm area appeared homogenous across the upper tape strips of all formulations. It was not until deeper tape strip layers where brighter spots of the CHG ion appeared, such as tape strip 12 from the 4% CHG-1mM PAMAM treatment group. Whilst CHG permeation appears heterogenous from tape strip 12 onwards, this was attributed to the decreasing amount of *stratum corneum* removed with increasing tape strip number, as observed and discussed previously31. The distribution of CHG within the *stratum corneum* is discussed further in **Section 3.3**.

**Figure 3** highlights how CHG ion intensity changes with SC depth as a function of tape strip number.

***Figure 3.*** *C7H4N2Cl- ion intensity values for tape strips 1, 2, 3, 6, 9, 12, 15, 18, 21 from treatment groups Hibiscrub****®*** *4% w/v, 4% w/v CHG and 4% CHG-1 mM PAMAM, (presented as mean ± SEM, n = 4). Areas normalised by total ion intensity. All pairwise comparisons indicate that a statistically significant result occurred (p <0.05). Abbreviation: CHG, chlorhexidine gluconate, PAMAM, polyamidoamine dendrimer.*

The results from **Figure 3** support those from the *in vitro* Franz-type diffusion cell study (**Figure 1C**). The CHG-PAMAM co-formulation increased CHG permeation compared to all other tape strips, and was considered statistically significant on tape strips 1-9 (p<0.05). Hibiscrub® 4% w/v consistently provided the lowest ion intensity values across all tape strips. The decrease in permeation of CHG as a function of increasing tape strip number is due to the depletion of a hydrophilic drug in a lipophilic environment: the log P of CHG is (0.013314) and the lipid component of SC is made up of cholesterol, free fatty acids and ceramides in a 1:1:1 ratio76. This drug depletion with increasing tape strip number is perpetuated by the limited release of CHG from a hydrophilic environment (aqueous gel) to a hydrophobic environment (skin surface), as the drug will preferentially remain in the formulation because of its physicochemical properties, which align with the aqueous gel.

This supports the notion that the G3 PAMAM-NH2 dendrimer is able to act as a permeation enhancer of CHG, increasing the concentration of CHG detectable in deeper skin layers able to target opportunistic, “hidden” flora. The creation of the novel co-formulation containing drug and dendrimer in this study illustrates that enhanced CHG deposition could realistically be implemented in practice, as the co-formulation provides a simple and convenient method of enhanced CHG delivery and thus is likely to be adopted by the end consumer.

From a clinical standpoint, application of the co-formulation immediately before surgery may not necessarily be the most suitable approach as application a few minutes before surgery (as is the case for Hibiscrub®) may delay depth permeation to the levels seen within this *in vitro* study after 24 h. However, this delay in opportunistic bacteria targeting may be overcome by providing the gel co-formulation containing 4% w/v CHG and 1mM G3 PAMAM-NH2 dendrimer for self-application at home, 24 h prior to surgery. This would allow CHG to permeate into the skin and reach deeply rooted opportunistic bacteria within the precise timeframe required. In addition, the enhanced permeation demonstrated by this formulation garners further application opportunities for the treatment of deeply rooted skin and soft tissue infections caused by microbial invasion into the skin and underlying tissue, which are typically caused by *Staphylococcus* and *β-haemolytic streptococci* 77.

**3.3 The Effect of CHG-PAMAM Dendrimer formulation on the *In Vitro* Permeation of CHG – Cross Section ToF-SIMS Analysis**

To further investigate the effect of the dendrimer on the intradermal delivery of CHG, ToF-SIMS analysis of cross-sectioned skin samples was employed. Understanding the spatial distribution of molecular species is paramount in elucidating the effectiveness of different drug delivery systems within a biological tissue. Conventional liquid chromatography mass spectrometry (LC-MS) is typically employed to understand the effectiveness of a drug delivery strategy, however the extraction process employed leads to a loss in spatial information. ToF-SIMS has the capability to simultaneously map secondary ions related to the dosed compound and ions from the native skin tissue45.

Three ions of interest were overlaid and coloured (C7H4N2Cl-, green; C5H11NPO4- red; C27H45SO4-, blue) to allow the secondary ion of CHG to be localised within the skin (**Figure 4**). C5H11NPO4- (phosphatidylethanolamine, a type of phospholipid)32 and C27H45SO4- (cholesterol sulphate) have previously been recognised as ions indicative of native skin components30. It is worth highlighting that the use of 0.3 pA pulsed target current may be considered as high for tissue analysis. Upon assessing the spectra and it is acknowledged that some peaks do appear saturated, especially at a low mass range such as Cl-, due to the high target current used. However, the use of a high target current was necessary as some of the peaks analysed were of a higher mass range. Thus, a higher target current was necessary for higher masses to achieve sufficient secondary ion intensity. This is of great importance for mapping datasets where high counts/pixel are desired, in order to have provide a suitable contrast for detecting and monitoring the drug within the skin samples. We have inspected the peak of interest C7H4N2Cl- (*m/z* 151); C5H11NPO4- (*m/z* 180) and C27H45SO4-, (*m/z* 465.3) by visualising changes in the peak before and after deadtime corrections as shown in **Figure S3**. It was apparent that there were changes to the Cl- peak before and after deadtime correction, highlighting the presence of peak saturation. However, for the peak of interest C7H4N2Cl- (*m/z* 151); C5H11NPO4- (*m/z* 180) and C27H45SO4-, (*m/z* 465.3), there were minimal changes to the peak before and after deadtime corrections, suggesting that there were minimal to no peak saturation for the higher mass range monitored.

***Figure 4. (a)*** *Negative polarity ToF-SIMS spectra of ex vivo porcine skin treated with 4% CHG-1 mM PAMAM, where the inset spectrum shows the peak of the chlorhexidine fragment ion at m/z=151, phosphatidylethanolamine fragment ion at m/z=180 and cholesterol sulphate molecular ion at m/z=465.3* ***(b)*** *ToF-SIMS 2D chemical ion maps of phosphatidylethanolamine fragment ion (dermis), chlorhexidine fragment ion (drug) and cholesterol sulphate molecular ion (stratum corneum, epidermis and hair follicle marker) acquired from cross section analysis of ex vivo porcine skin tissue after a 24 hour permeation experiment. Chemical ion map shows location of skin tissue along with the biodistribution of active and native skin chemistry. The overlay indicates the ability ToF-SIMS to detect the localisation of chlorhexidine (active) within the ex vivo skin tissue in a label free manner. Abbreviation: CHG, chlorhexidine gluconate, PAMAM, polyamidoamine dendrimer. Scale bar: 500 µm*

The spatial distribution of CHG is clearly visible in **Figure 5(A)**. Furthermore, the image analysis of cross sections of the skin allows one to gauge the drug distribution within all the skin layers as opposed to the tape stripping study, which focused on the drug distribution within the SC only.

***Figure 5.******(a)*** *Comparative ion intensity overlain chemical distribution maps illustrating the chemical distribution of C5H11NPO4- (phosphatidylethanolamine fragment ion, red), C7H4N2Cl- (chlorhexidine fragment ion, green) and C27H45SO4- (cholesterol sulphate fragment ion, blue) across untreated skin, Hibiscrub® 4% w/v, 4% w/v CHG, and 4% CHG-1 mM PAMAM. Each image represents 1.5 mm × 3 mm area analysed.* ***(b)*** *Depth of permeation of CHG (C7H4N2Cl- ion) from Hibiscrub® 4% w/v, 4% CHG gel and 4% CHG-1mM PAMAM gel (presented as mean ± SEM, n = 10), were measured using the line scan function in the measurement editor tool in Surface Lab software. The translucent grey box indicates the 400-700 µm depth target of CHG permeation. Abbreviation: CHG, chlorhexidine gluconate, PAMAM, polyamidoamine dendrimer. Scale bar: 500 µm*

Untreated skin showed no observable intensity for the CHG ion (C7H4N2Cl-, green colour, **Figure 5(A**). The C5H11NPO4- ion successfully showed theviable epidermis and below (red colour), with a clear darker band above which indicated the presence of the SC where there was little C5H11NPO4- ion intensity. The cholesterol ion (C27H45SO4-, blue colour), which has a higher intensity in the SC and epidermis, but displays a lower ion intensity in the deeper epidermal layers was used to identify the presence of the SC and epidermis. Such observations on the localisation of C27H45SO4- to the SC and upper epidermis regions is further corroborated by the findings by Sjövall *et al.*78 who also observed similar ion distribution in human skin cross sections. Together, the combination of the C5H11NPO4- ion and C27H45SO4- ion indicated the epidermal-dermal junction. This is clear when viewing the untreated skin samples without interference from the unique CHG ion fragment (C7H4N2Cl-).

The most prominent change was apparent when comparing the formulations with and without the PAMAM dendrimer. For formulations without the addition of the permeation enhancer, there appears to be very little CHG intensity within the epidermis and below as the C5H11NPO4- ion (red colour) is still clearly visible from the overlay images. However, the formulation that contained 1 mM PAMAM, indicates that a clear CHG ion intensity gradient permeates into the epidermis and was even present within the dermis. The 4% CHG-1 mM PAMAM was found to deliver CHG to a depth of 825.09 ± 157.10 µm, therefore into and beyond the 400-700 µm zone cited as an indicator of appendages which have been thought to hold reservoirs of bacteria (**Figure 5(B)**)79, without permeation through full thickness porcine skin. This increase in depth permeation was considered statistically significant when compared to all other formulations measured (p <0.05). The depth permeation measurements using the “line scan” and “measurement editor” IONTOF tool may only act as support for other types of data due to the manual application of the tool and thus the subjectivity of depth permeation. Nonetheless, it is abundantly clear from **Figure 5A** that the addition of the PAMAM dendrimer at a concentration of 1 mM was able to enhance CHG depth permeation.

The cross section images following a 24 h treatment with Hibiscrub® 4% w/v appear to show a large area of green colouration indicating the CHG ion. It should be noted that this is not representative of CHG permeation into the SC, but is caused by smearing of the cross section when transferring the sample during the thaw mounting step prior to ToF-SIMS analysis, and was likely due to differences in formulation increasing the risk of smearing. This highlights the need for the C5H11NPO4- and C27H45SO4- ions to co-localise the CHG ion that has specifically permeated into the SC. The depth permeation of Hibiscrub® 4% w/v into porcine skin was measured as 39.41 ± 22.27 µm (**Figure 5(B**).

In **Figure 2**, the permeation of CHG within the *stratum corneum* appeared uniform until tape strip 12 onwards. The patchiness of CHG distribution thereafter was attributed to the inability of tape strips to remove intact corneocytes from deeper skin layers, as discussed previously31. This was likely due to the increased prevalence of lipid packing and corneocyte presence in the deeper skin layers, in addition to the increased amount of interstitial fluid present in deeper skin layers which prevents adequate tape strip adhesion to the skin samples. This unfortunate disadvantage of the tape stripping technique supports the use of ToF-SIMS analysis of CHG treated cross sections to provide an accurate picture of CHG distribution across the *stratum corneum* (**Figure 5**). CHG permeation appears homogeneous across the entirety of the *stratum corneum*in **Figure 5**, and together with the information provided from **Figure 2,** implies that CHG distribution across the skin is relatively homogenous and thus potentially pathogenic skin bacteria may be targeted across the entirety of skin, rather than being limited to specific skin sites, folds or furrows.

This implication is confirmed in **Figure S4**, which clearly illustrates the presence of a sweat gland (**S4(A)**) and hair follicle root (**S4(B)**) without CHG permeation. The physicochemical properties of the drug dictate that the drug is more likely to permeate into skin via the transcellular and or transappendageal routes (i.e. low log P of 0.013314, molecular weight 897.8 g/moL12 and ionisation state at physiological pH). The role of follicles in transdermal drug delivery is contentious. It is argued that polar molecules, such as CHG, may be able to diffuse dermally via the appendages80,81. However, this does not take into account the fact that follicles are lined with cornified cells, and follicles are often filled with sebum82. Therefore, a lack of CHG permeation into the hair follicle may be attributed to the extension of the SC into the follicle, and potentially the presence of sebum in the follicle, providing a lipophilic environment in which CHG would struggle to permeate into. The presence of “open” and “closed” follicles was confirmed by Otberg *et al.*83, where plugs of shed corneocytes pushed out of the follicle orifices by growing hairs or emerging sebum could prevent the ingress of exogenous chemicals84. Furthermore, the apparent lack of appendageal permeation may be due to intraspecies variation. In addition, CHG may permeate across the thin aqueous pathway between the polar head groups of the lipid lamellae, so it is possible this route was also utilised by the drug for partitioning into skin. This route avoids the requirement of repeated partitioning between lipid and aqueous phases required by the transcellular route, so is usually preferred by hydrophilic compounds where possible, despite the tortuosity of this route.

Skin is not homogenous, thus characterising this complex structure and accurately quantifying the depth permeation of a drug is difficult, though the OECD guidelines on *in vitro* skin absorption18,19 provides the most appropriate guidance for *in vitro* studies. Though ToF-SIMS cannot be considered a replacement for traditional HPLC extraction and analysis, as results can only be considered semi-quantitative, the use of this technique may provide useful complementary information, providing accurate intradermal depth permeation measurements (**Figure 5**), allowing co-localisation of a drug alongside endogenous skin components (**Figure 4**), and presenting information on the drugs permeation pathway (**Figure S4**).

The cross section images further support the results obtained from the tape stripping study (**Figure 2**). To summarise, CHG without the addition of a permeation enhancer strongly binds to the upper skin layers85 with poor permeation into deeper tissues. A clear gradient is visible from the 4% CHG-1 mM PAMAM formulation treated skin where the CHG is able to permeate past the SC into deeper skin tissues (**Figure 5**). The cross sections additionally showed that the optimised co-formulation in the presence of the PAMAM dendrimer delivered CHG into the dermis, to the depth proposed by Touitou, Meidan and Horwitz9 as the depth of inaccessible bacteria. The results of this study therefore indicate that a convenient, practical and user friendly gel was formulated with the ability to enhance CHG permeation compared to the commercial benchmark, Hibiscrub®. By targeting potentially pathogenic bacteria in these deeper skin layers, one may expect a reduced risk of skin and soft tissue infections in those undergoing surgery, particularly in at risk groups such as the elderly or immunocompromised. However, due the discrepancies between experimental and real-world conditions, the duration of application prior to surgical intervention may serve as a translational barrier. One way of circumventing this is issue is to provide patients with the co-formulation for self-application at home 24 h before surgery to allow CHG to permeate into the skin and reach the deeply rooted opportunistic bacteria in the skin, thus reducing the propensity of post-surgical infections.

Collectively, the data shows that PAMAM dendrimers are an elegant drug delivery system, able to improve the intradermal delivery of an existing, first line antimicrobial for the purposes of targeting potentially pathogenic, “hidden” bacteria. The novel gel formulation co-formulated with the G3 PAMAM-NH2 dendrimer in this study enables the drug to be reformulated into a simple, user friendly gel, enabling ease of commercialisation and translation into clinical practice.

**4.0 Conclusion**

In conclusion, this study effectively demonstrated the ability of a gel formulation containing 4% w/v CHG and 1mM G3 PAMAM-NH2 dendrimer to enhance the depth permeation of CHG within porcine skin. Permeation from the optimised co-formulation was found to deliver CHG across the entirety of the dermis, without apparent permeation *via* specific routes, such as appendages. The permeation enhancement effect of the PAMAM dendrimer was attributed to a physical effect on the skin barrier as opposed to a specific interaction with the drug, most likely occlusive effects. The optimised co-formulation demonstrated an improvement in CHG depth (825.09 ± 157.10 µm from the co-formulation compared to 39.41 ± 22.62 for the commercial benchmark) whilst providing a convenient and practical method of CHG delivery. The formulation of a simple and practical one-step gel increases the likelihood of clinical translation and its acceptance by the end user, and in increasing the efficacy of CHG, reduces the need for the development of new antimicrobials to combat the predicted, imminent antimicrobial crisis.

**Supporting Information**

**Figure S1.** Illustration of the “Measurement Editor” feature using the IONTOF software. Depth permeation of the C7H4N2Cl- ion within porcine skin cross sections are measured manually and are illustrated by the green line.

**Figure S2. A.** Chemical structure of CHG. **B.** Structure and m/z of CHG fragment used to map drug depth permeation using ToF-SIMS, previously discovered by Holmes et al.1. **C.** Mass spectra indicating specificity of C7H4N2Cl-ion to CHG (green peak) which is only present on tape strips taken from skin treated with CHG formulation. **D.** Tape strip images indicating specificity of C7H4N2Cl- ion to CHG. Each image represents a 4 mm × 4 mm area.

**Figure S3.** Changes in peak shape before (grey line) and after (red line) dead-time correction. Changes to Cl- peak before and after deadtime correction highlights the presence of peak saturation. However, for the peak of interest C7H4N2Cl- (m/z 151); C5H11NPO4- (m/z 180) and C27H45SO4-, (m/z 465.3), there were minimal changes to the peak before and after deadtime correction, suggesting there were minimal to no peak saturation.

**Figure S4. A.** Suggestion of a sweat gland (PO3-) with lack of CHG permeation (indicated by white arrows). **B.** Suggestion of a hair follicle (C27H45SO4-) with lack of CHG permeation (indicated by white arrows).

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