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Ex-vivo measurement of scalp follicular infundibulum delivery of zinc pyrithione and climbazole from an anti-dandruff shampoo

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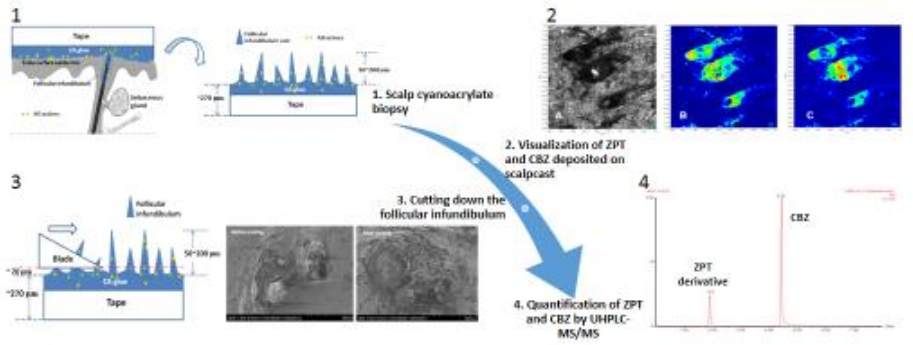
Graphical abstract



Highlights

- A cyanoacrylate biopsy based method is developed to sample the zinc pyrithione (ZPT) and climbazole (CBZ) deposited onto the scalp surface and follicular infundibula.
- Spatial distribution of ZPT and CBZ deposited on scalp is visualized by Raman imaging.
- A tailor made cutting device enables the separation scalp surface sample from scalp follicular infundibulum samples.
- Follicular infundibulum delivery of ZPT and CBZ from an anti-dandruff shampoo is determined.

Abstract

Efficient delivery of anti-dandruff (AD) actives into the scalp follicular infundibulum as well as onto the scalp surface is critical for the efficacy of AD shampoos. A method involving scalp cyanoacrylate (CA) biopsy sampling, a tailor made cutting device, ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) analysis, scanning electron microscopy (SEM) measurement and Raman imaging has been developed for the measurement of

delivery of zinc pyrithione (ZPT) and climbazole (CBZ) from an AD shampoo into the scalp follicular infundibulum. Scalp CA biopsy enables the sampling of ZPT and CBZ delivered into the scalp follicular infundibulum as well as onto the scalp surface. Raman imaging of scalp CA biopsy samples allows the visualization of the spatial distribution of ZPT and CBZ deposited on the scalp. A tailor made cutting device enables the separation of the scalp follicular infundibulum sample (20 μm below the scalp surface) from the scalp surface samples (including top 20 μm of infundibula). UHPLC-MS/MS was used as a sensitive and specific methodology enabling the quantification of ZPT and CBZ without interference. Using this method, both ZPT and CBZ were successfully quantified and spacially visualized within the scalp follicular infundibulum, after scalp was washed with an AD shampoo.

Keywords

Zinc pyrithione, climbazole, anti-dandruff, scalp follicular infundibulum delivery, cyanoacrylate biopsy and clinical study

1. Introduction

Dandruff is a common scalp issue which affects approximately 50% of the global population and is characterized by flakes and itch on the scalp without visible inflammation [1-3]. Overgrowth of *Malassezia*, a genus of lipophilic yeasts was reported to be associated with dandruff [4-7]. Hence, one of the most common treatments of dandruff is the use of an anti-dandruff (AD) shampoo containing antifungal agents like zinc pyrithione (ZPT) and climbazole (CBZ) [8, 9].

It is known that *Malassezia* yeasts live on human skin around the opening of the hair follicles (also called infundibulum) as well as on the scalp surface [5,10,11]. Schwartz and his team claimed that efficient delivery of AD actives into the scalp follicular infundibulum as well as onto the scalp surface is critical for the efficacy of AD shampoos [12,13]. Reliable analytical methods are required for the measurement of spatial delivery of AD actives on the scalp. However, besides buffer scrub, cyanoacrylate (CA) biopsy, tape stripping and hair plucking, very limited sampling methods have been developed for detecting AD actives on the scalp after the application of AD shampoos.

In previous studies [14,15], we developed methods for the measurement of ZPT and CBZ deposition on scalp from AD shampoo using buffer scrub as the sampling method. However, the methods are not able to distinguish between scalp surface delivery and follicular infundibular delivery. An *in vivo* imaging method using confocal microscopy was reported to enable the measurement of ZPT spatial distribution in the scalp follicular infundibula [13]. Confocal microscopy imaging can detect ZPT particles optically but not chemically. When other similar particles are delivered together with ZPT, chemical imaging tools like Raman imaging are demanded to offer chemical specificity, so as to eliminate the impact of other particles.

Since Marks and Dawber first used CA for skin surface biopsy in 1971 [16], CA based sampling methods have been further developed and proven to enable both skin surface and

follicle biopsies. Combined with imaging technologies, cyanoacrylate skin surface stripping (CSSS) has been widely applied in diagnostic dermatopathology and cosmetology, as well as in experimental dermatology settings [17]. To investigate the penetration of topically applied substances into hair follicles, Teichmann and his team developed a differential stripping method which was based on CSSS [18,19]. For measuring ZPT delivery into the scalp follicular infundibula, a sampling method which combined CA biopsy and hair pluck was reported [13]. In this method, a drop of CA glue was applied to the infundibulum of a hair follicle. After drying, the CA glue together with hair(s) was removed, obtaining a follicular cast containing cell debris, sebum, hair(s), ZPT etc. To make the analysis be representative for the whole hair follicles, the sampling should cover multiple follicles.

The aim of this study was to develop a method to measure scalp follicular infundibulum delivery of ZPT and CBZ onto the scalp from a dual-active AD shampoo. A CA based *in vivo* sampling method was developed to harvest ZPT and CBZ delivered on both the scalp surface and in follicular infundibula. After the sampling, scalp CA biopsy samples (casts) were subjected to Raman and scanning electron microscopy (SEM) imaging. A tailor made cutting device was developed to efficiently and accurately separate the scalp surface casts (SCs) from scalp follicular infundibulum casts (FICs) at a defined z-distance. After separation of the layers, the contents of ZPT and CBZ in scalp SCs and FICs were determined by an ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) method.

2. Materials and methods

2.1 Chemicals and reagents

All reagents and solvents used in the experiment were analytical grade or above. CBZ (99.9%), ZPT (95%), and 2,2-dipyridyl disulfide (DPS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate and EDTA-2Na were purchased from SCRC (Shanghai, China). HPLC grade acetone, methanol and acetonitrile were purchased from Merck (Darmstadt,

Germany). Milli-Q pure water (18.2 MΩ, Millipore, Bedford, MA, USA) was used to prepare samples, standard solutions and UHPLC mobile phases. Model sebum was prepared by mixing the lipid compounds purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Test shampoos

A commercial beauty shampoo without AD actives was used as run-in shampoo. The test shampoo used in this study was commercial dual-active AD shampoo containing ZPT and CBZ.

2.3 *In vivo* clinical study and CA biopsy sampling

The *in vivo* clinical study was a randomized, double blind and controlled study. The study was conducted in China and was reviewed and approved by the ethical committee of the Shanghai Clinical Research Center. Informed consent was obtained from all subjects before participation. Healthy subjects of both gender aged 18-60 years were screened and had their scalp condition visually assessed using Unilever Total Weighted Head Score (TWHS) system [3]. Subjects with scalp condition of whole head TWHS adherent flake (AF) ≤ 8, with no flake grade B or above, were accepted. Subjects who had used shampoo containing anti-dandruff actives within the last 4 weeks were excluded. Forty five subjects were accepted and 43 subjects (24 females + 19 males) completed the whole study. Two subjects were withdrawn for personal reason. No products or procedure related adverse event was reported.

All subjects had their hair washed using beauty shampoo 2 days before the test shampoo wash. Then subjects had their hair washed using the same dose of the dual-active AD Shampoo and blow-dried following standard hair wash operation. Square regions of 1 cm×1 cm were identified as sampling sites for scalp CA biopsy. Hair in this region was carefully cut from the root as close to the scalp skin as possible and operation contact with scalp skin was minimum.

A small amount of CA glue (Loctite Medical grade CA glue 4061) was applied onto the rough side of the Melinex® sampling strip and spread evenly to give a thin film. Afterwards, the glue-coated strip was applied to the sampling area and pressed firmly onto the scalp. The strip sample

was peeled off when dry. All CA scalp biopsy samples were stored in an air-tight container before further analysis. Figure 1 shows how CA scalp biopsy is used to obtain the FICs and SCs consisting of cell detritus, lipids, microbial and AD actives.

2.4 SEM measurement

The scalp CA biopsy samples before and after cutting were sputter-coated with platinum using an ion sputter coater (E-1045, Hitachi, Tokyo, Japan) for 90 seconds with a current of 15 mA. The SEM images were taken by using a SEM (Hitachi S-4800) to measure the size of FIC. Imaging conditions were set as follows: high voltage = 5 or 10 kV; beam current = ~10 μA; working distance = 5-10 mm; magnification = 30 × – 20,000 ×.

2.5 Raman measurement

A Raman microspectrometer (Horiba Jobin Yvon, HR Evolution, Kyoto Japan) was employed to visualize the spatial distribution of CBZ and ZPT on scalp CA biopsy samples before cutting. Pure materials of CBZ, ZPT, CA glue and model sebum were used as reference materials to assign signals in the obtained Raman spectra. On each sample, one region in size of ~ 2 mm × 2 mm (containing at least 3 follicles) was scanned under a 10x air objective with a 532 nm laser source. The step size was 10 μm (~ 40, 000 pixels), and spectra in each pixel was taken from 400 cm^{-1} to 2000 cm^{-1}, with 0.5 second laser exposure with 2 times accumulation to remove cosmic rays. The resultant spectra matrix was analyzed by built-in direct classical least square (CLS) fitting algorithm to demonstrate the relative distribution of each active.

2.6 Separation of scalp FICs from scalp SCs

After sampling, the scalp CA biopsy samples were cut by a tailor made cutting device which was developed in house. The principle of cutting down the follicular infundibulum cast is shown in Figure 2. Before cutting, the blade height of the cutting device was carefully adjusted to 20 μm, so as to only cut down the FICs without SCs. Scalp CA biopsy samples are placed on a weighing

paper. Then the cutting device was pushed forward horizontally to sweep over the sampling area. This was repeated several times. The obtained FICs were so tiny that they should be carefully collected from the tape as well as from the blade. After cutting, the white ash was transferred into a tube pre-marked "scalp infundibulum sample". The residual CA biopsy sample was collected as "scalp surface sample", which included the top 20 μm infundibulum.

2.7 Quantification of ZPT and CBZ by UHPLC–MS/MS analysis

A Waters ACQUITY UPLC system coupled to a Quattro Micro API mass spectrometer (Waters, Manchester, UK) was used for the quantitative analysis of ZPT and CBZ. Both "scalp infundibulum samples" and "scalp surface samples" were subjected to acetone extraction. A standard stock solution of ZPT and CBZ was prepared in methanol at a concentration of 2 mg mL^{-1} each. The working solutions were prepared freshly by the appropriate dilution of the stock solution with a mixture of 50% of mobile phase A (20 mM ammonium acetate in water) and 50% mobile phase B (methanol). Following the previously published method [13], both sample extraction and standard solutions were subjected to DPS derivatization prior to UHPLC-MS/MS analysis. The delivery levels of ZPT and CBZ were expressed as ng/cm^2.

3. Results and discussion

3.1 SEM imaging of scalp CA biopsy samples

The infundibulum is the upper segment of the hair follicle, extending from the surface to the sebaceous gland. The total length of the infundibulum is approximately 500 μm [20]. SEM images (Figure 3) of scalp CA biopsy samples visualize the morphology of a scalp FIC which look like hollow cones without tips. The hollow cone was caused by residual hair shafts in follicles, although hair was clipped to scalp level prior to sampling. The dimensions of the FICs (height and diameter) were measured by the build-in ruler function of the SEM. The FICs heights were in the 50-200 μm range, which indicates that scalp CA biopsy enables a sampling depth of

200 μm in the follicular infundibula. The diameter at the base of FICs ranged from 100 to 250 μm. All scalp CA biopsy samples were measured by SEM and images were studied individually to verify that infundibulum casts had been successfully collected.

3.2 Spatial distribution of ZPT and CBZ on scalp CA biopsy samples

Pure CA glue, CBZ, ZPT and model sebum were used as reference materials to obtain standard Raman spectra (Figure 4). A multivariate curve resolution (MCR) algorithm (CLS) [21] was used for spectra interpretation and image reconstruction. For each image, at least three FICs were measured. Figure 5 shows a typical Raman image of a scalp CA biopsy sample, which indicates that both ZPT and CBZ were delivered more into the follicular infundibula than onto the scalp surface when applyling the dual-active AD shampoo. Most likely during rinse-off, the ZPT and CBZ on the scalp surface were more easily rinsed-off than that present in the follicular infundibula. Current Raman images enable the visualization of ZPT and CBZ distribution on scalp.

3.3 Separation of scalp FICs from scalp SCs by a cutting device

A tailor made cutting device was designed and made for cutting down the scalp FICs. It is constructed of a vernier caliper and a blade. The vernier caliper enables easy tuning of the blade height with a minimum tuning step size of 1 μm. The blade is circular, which ensures the blade edge always faces the casts after adjusting the blade height. Efficiency of the follicle cutting device was verified by SEM images of the scalp CA biopsy samples before and after cutting (Figure 6). The blade height away from surface is critical for complete separation of FICs from SCs. Based on the surface morphology of CA biopsy samples, the blade height was set at 20 μm in this study. This setting ensured that pure FICs were cut off without any SCs. As a result, however, some FICs (less than 20 μm height) remained on the CA tape and were treated as scalp surface samples. After cutting, the scalp CA biopsy samples were divided into scalp

infundibulum samples (deeper than 20 μm) and scalp surface samples (including top 20 μm of infundibula).

3.4 Deposition levels of ZPT & CBZ into scalp follicular infundibula

The contents of ZPT and CBZ in scalp infundibulum samples and scalp surface samples (shown in Table 1) were quantified by the UHPLC-MS/MS method. The method is sensitive enough to detect ZPT and CBZ at ppb level (corresponding to 5 ng/cm^2). More ZPT and CBZ were detected in scalp surface samples (ZPT, 2770 ± 2540 ng/cm^2 and CBZ, 550.1± 270.5 ng/cm^2) than in scalp infundibulum samples (ZPT, 11.0 ± 9.0 ng/cm^2 and CBZ, 10.3 ± 9.5 ng/cm^2). This finding looks inconsistent with the Raman imaging results in which more ZPT and CBZ were observed in the follicular infundibula than on scalp surface. A study on the depth profile of scalp infundibular ZPT [13] observed a sharp drop-off of the amount of ZPT as the depth increases with the majority of ZPT (>80%) being present in the upper part of the infundibula (<20 μm). Due to the limitation of the cutting in the current method, the scalp surface samples included the top 20 μm of infundibula. Consequently, analysis of the harvested FIC underestimates the total ZPT delivery to the entire infundibulum. Another reason for the difference in findings between Raman imaging results and deposition levels is that the combined area of scalp follicular infundibula is dramatically smaller than that of the scalp surface [22-24].

The dual-active AD shampoo delivered almost the same levels of ZPT (11.0 ± 9.0 ng/cm^2) and CBZ (10.3 ± 9.5 ng/cm^2) into the infundibulum. However, a comparison of the ratio of infundibulum delivery (20 μm deeper than surface) *vs* total deposition between CBZ (1.80 %) and ZPT (0.40 %) suggests that CBZ penetrates deeper into follicular infundibulum than ZPT, suggesting that CBZ may be up to 4x more efficient at “targeting” the follicular infundibulum. The reason for this difference is likely due to CBZ having a higher solubility in sebum versus ZPT.

4. Conclusions

For the measurements of follicular infundibulum delivery of ZPT and CBZ from a dual-active AD shampoo, a method involving scalp CA biopsy sampling, FICs cutting, UHPLC-MS/MS analysis SEM measurement and Raman imaging has been developed. Scalp CA biopsy enables the sampling of ZPT and CBZ delivered into the scalp follicular infundibulum. Raman imaging of scalp CA biopsy samples allows the visualization of the spatial distribution of ZPT and CBZ deposition on the scalp. The cutting device enables the separation of scalp FICs from scalp SCs and the contents of ZPT and CBZ can be quantitated by the sensitive UHPLC-MS/MS analysis. The method detection limit allows the quantification of ppb levels of CBZ and ZPT delivered onto the scalp surface and into the follicular infundibulum (corresponding to 5 ng/cm^2).

Using this method, ZPT and CBZ delivered into the scalp follicular infundibulum (20 μm lower than scalp surface) from the dual-active AD shampoo was successfully visualized and quantified. Due to the lipophilic natue of CBZ and subsequent increased solubility in sebum, CBZ has the ability to penetrate further into the sebum-rich infundibulum whereas ZPT remains within the upper 20 μm of infundibula. This differential distribution of actives allows for the effective targeting of *Malassezial* species throughout the depth of the scalp follicular infundibulum.

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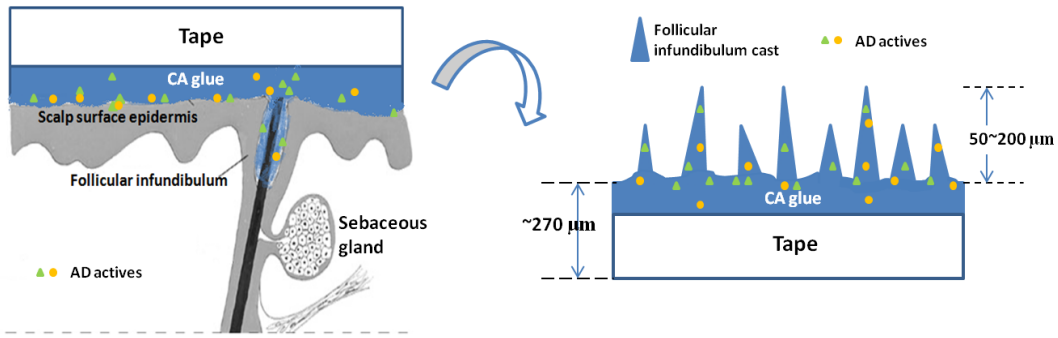

Fig.1. Principle of scalp CA biopsy sampling

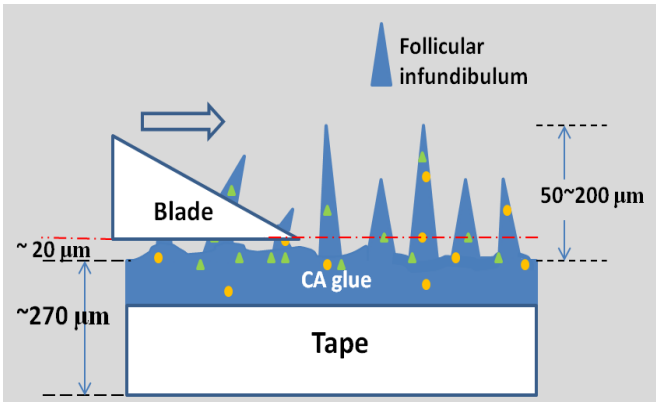

Fig.2. Principle of cutting down the follicular infundibulum cast.

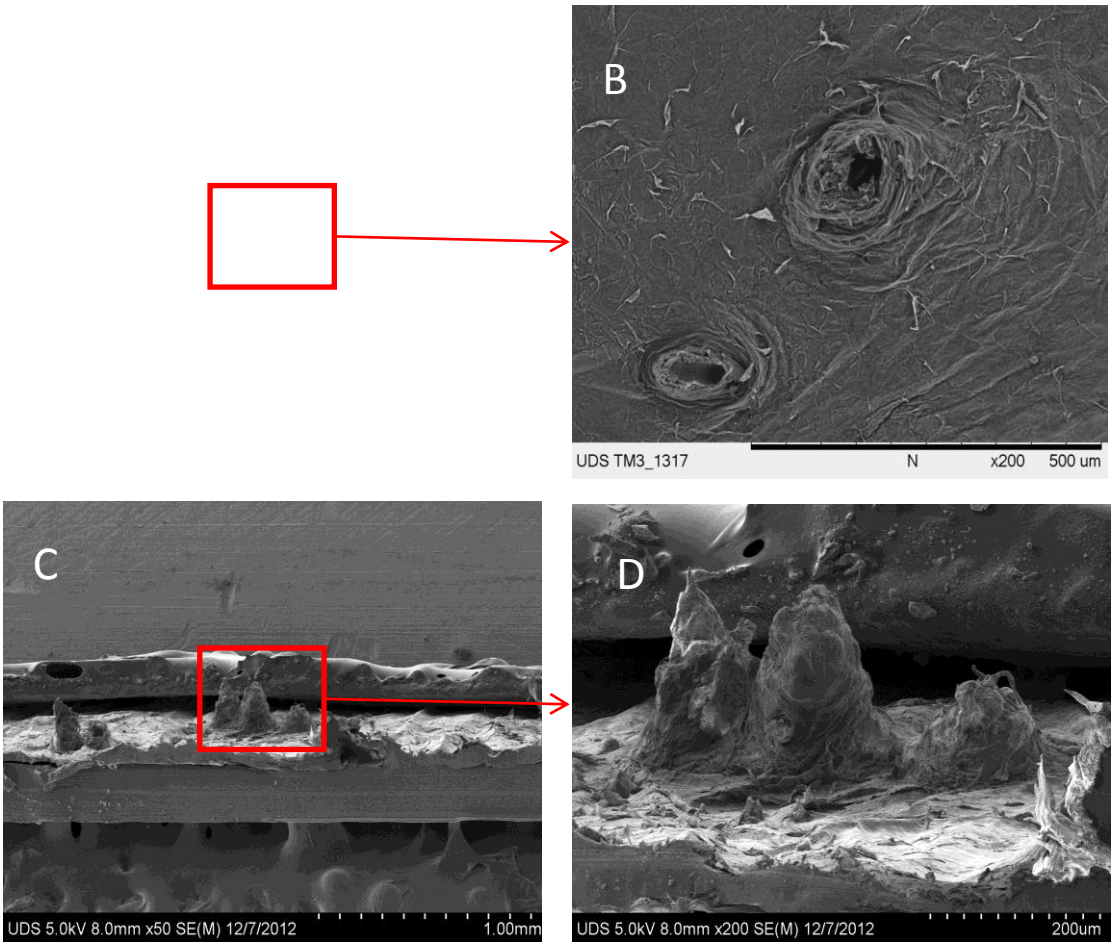

Fig.3. SEM images of scalp CA biopsy samples.

A and B: top view images of a representative scalp CA biopsy sample; C and D: cross-sectional view of a representative scalp CA biopsy sample at different magnifications.

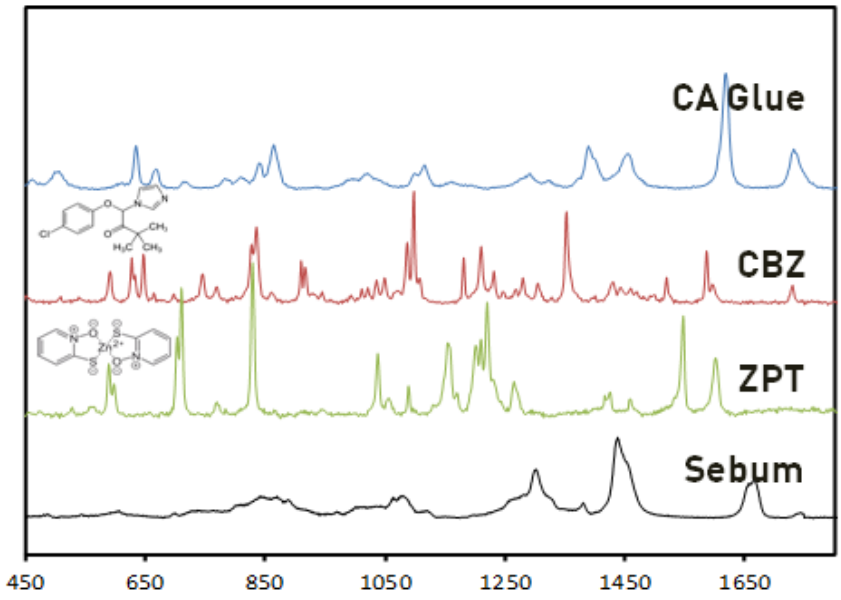

Fig. 4. Raman spectra of CA glue, CBZ, ZPT and sebum.

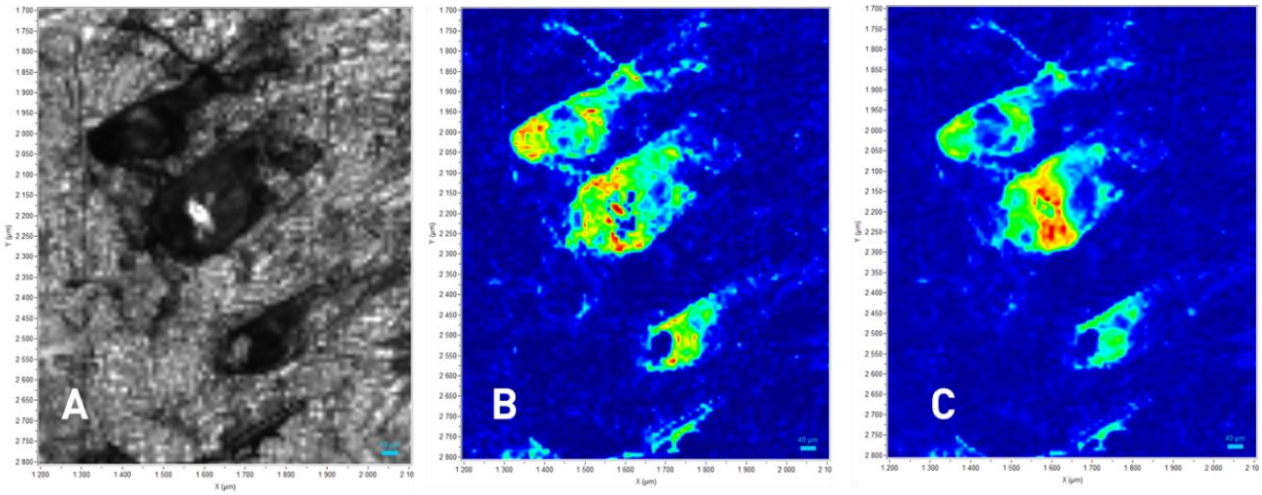

Fig. 5. Raman images of a representative scalp CA biopsy sample. (Scale bars indicate 40 μm).

A: Mapping of CA glue (in grey scale); B: Mapping of CBZ; C: Mapping of ZPT (Red color shows higher intensity and blue color shows lower intensity).

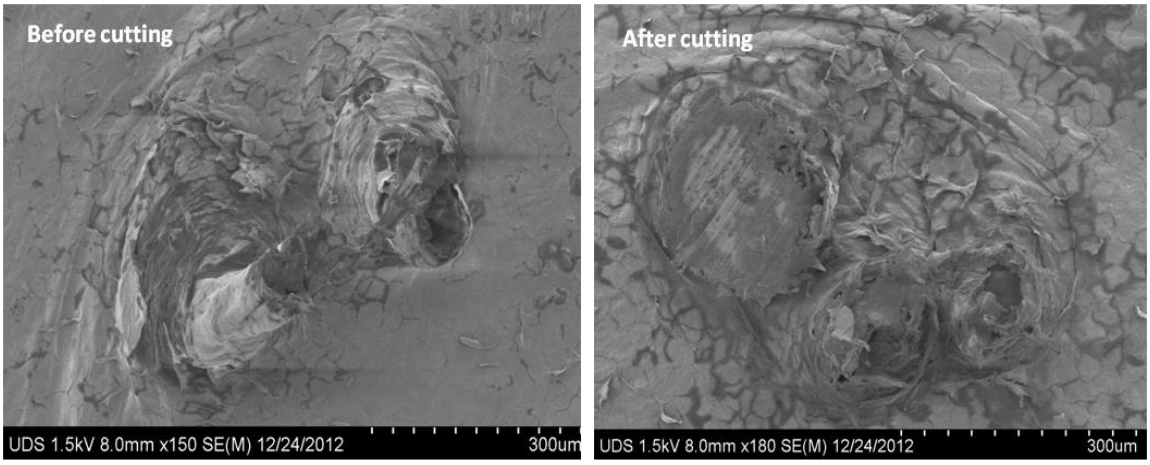

Fig.6. SEM images of scalp CA FICs before cutting (left) and after cutting (right).

Table 1. Scalp surface and follicular infundibulum delivery of ZPT and CBZ from the dual-active shampoo.

AD active deposition from the dual-active AD shampoo	**ZPT**	**CBZ**
Scalp surface samples, ng/cm^2 (including top 20 µm infundibulum)*	2770.0 ± 2540.0	550.1± 270.5
Scalp infundibulum samples, ng/cm^2 (20 µm deeper than surface)*	11.0 ± 9.0	10.3 ± 9.5
Total deposition, ng/cm^2	2781 ±2547.0	560.4 ± 277.0
Ratio of infundibulum delivery *vs* total deposition	0.40%	1.80%

* Mean ± Standard Deviation, n=60.