Electron microscopy-image analysis: Quantification of ultrastructural changes in hair fiber cross sections as a result of cosmetic treatment

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Synopsis
In a recent publication, Kaplin et al. described the dissolution of ultrastructural components in hair fibers as a direct result of various cosmetic treatments. These observations were based on a subjective assessment of holes or voids observed in electron micrographs of hair fiber cross sections. We have further investigated these effects using electron microscopy in conjunction with image analysis. Cross sections of proximal and distal ends of intact hair fibers were examined before and after repeated shampooings. The total number of holes, total projected areas, mean areas, and size distributions were determined in the cuticle and cortex regions of hair fibers using a Quantimet 900 Image Analyzer. All measured parameters indicated that ultrastructural disruption increased from the proximal to the distal end of hair fibers. The impact of shampooing was limited to the cuticle region of weathered hair fibers.

INTRODUCTION
The ultrastructural organization of the hair fiber has been extensively studied over the past decade. The keratin components of the hair, which constitute about 85% of the mass of the fiber, are considered to make the major contribution to the chemical and physical properties of the hair. The remainder of the fiber consists of non-keratinous materials in the form of intercellular membranes and the remnants of cellular constituents. These non-keratinous materials have recently been the subject of investigations concerning their contribution to the integrity of the fully keratinized hair fiber.

Figure 1 provides a schematic diagram of the non-keratinous regions of the hair fiber in transverse section. The detailed structure and chemical composition of these regions have been revealed by electron microscopy used in combination with various chemical studies.

The intercellular membranes form a network structure between both cuticle and cortical cells. Electron micrographs show that these membranes consist of two lipid layers, the β-layers, and a central non-keratinous protein layer, the δ-layer, often referred to as the intercellular cement (1–3). The cell membranes and the intercellular cement make up the so-called cell membrane complex of keratin fibers. Although resistant to attack
from proteolytic enzymes (4), this complex is disrupted by organic solvents such as formic acid (5). Disruption of these membranes ultimately results in a complete breakdown of fiber structure.

Another major non-keratinous component is the endocuticle which is located at the inner portion of each cuticle layer. The endocuticle represents the remnants of the once viable cuticle cell. In the cortex, analogous components are the nuclear remnants and intermacrofibrillar matrix. These components, unlike the cell membrane complex, are composed primarily of protein and are readily digested by proteolytic enzymes. Using electron micrographs, Swift and Bews (6) showed that the liberation of cuticle cell-like units by proteolytic enzymes is due to digestion along the endocuticle sheet rather than splitting of the cell membrane complex. Similarly, they suggested that cortical cells might be liberated by digestion along the intermacrofibrillar matrix. These studies suggest that the non-keratinous components play a significant role in acting as a cement to hold the keratin fiber together.

More recently, the vulnerability of these non-keratinous components to cosmetic treatments has been investigated. Using electron microscopy, Mahrle et al. (7) showed the loss of substances from the endocuticle after treatment with cold-waving solutions. Similarly, Kaplin et al. (8) reported that repetitive shampooing and drying treatments resulted in the dissolution of material from the endocuticle of hair fibers. These observations were based on a subjective assessment of holes or voids appearing in electron micrographs of hair fiber cross sections. Electron microscope studies in our laboratory indicate that endocuticular voids as well as other forms of ultrastructural disruption are present in cross sections of hair fibers subjected to normal grooming processes (Figure 2).

We have further investigated the effects of cosmetic treatments on the ultrastructure of hair using electron microscopy in conjunction with image analysis. Our first objective was to develop a technique which would quantitatively assess ultrastructural changes
and then use this technique to study the effects of weathering and shampooing on the non-keratinous components of the hair.

MATERIALS AND METHODS

HAIR SAMPLING

Brown hair fibers were obtained from one female subject whose hair had not been treated with any chemically reactive cosmetic products. Twenty fibers, each approximately 18 cm in length, were cut as close as possible (1 to 2 mm) to the scalp. All hair fibers were sampled from a mid-region in the back of the head.

SHAMPOO TREATMENT

After sampling, the hair was divided equally into two tresses of ten fibers each which were secured at the root end. The tresses were then clipped to a one-gram laboratory hair tress for shampoo treatment. The shampooing treatment consisted of a double lathering with a conventional anionic shampoo followed by air drying. One tress was shampooed through 90 shampoo cycles during a one-week period. This multiple shampooing treatment was designed to simulate approximately six months of on-the-head shampooing (3.5 shampoos per week). The second tress was shampooed through one shampoo cycle and was retained as a non-shampooed control.
Sample preparation for electron microscopy consisted of staining the tresses for two hours in 1.3% OsO4 in 0.067 M cacodylate buffer. Hair segments, 1 cm in length, were cut from the root and tip regions and embedded in Spurr's resin. Transverse sections approximately 120 nm thick were cut with a diamond knife in an LKB Ultratome NOVA ultramicrotome and collected on 200-mesh copper grids containing a formvar-carbon support film. The grids were then post-stained with uranyl acetate and lead citrate for image enhancement. A thin layer of carbon was evaporated onto the specimens to reduce electrostatic charging in the electron microscope.

The specimens were examined in an Amray 1200B scanning electron microscope operated in a transmission mode at 30 Kv with a final objective aperture of 200 μm. Photomicrographs were taken with Polaroid Type 52 black and white film. A total of 160 photomicrographs were taken at random of the cuticle and outer portion of the cortex at a final print magnification of 5000.

Image analysis of electron micrographs

The image analysis process consisted of, first, defining the types of ultrastructural changes observed. The cuticle and cortex regions were analyzed separately. In the cuticle, round holes or voids originating in the endocuticle were differentiated from linear voids which occurred between cuticle layers. In the cortex, irregularly shaped voids were analyzed.

A Quantimet 900 Image Analysis System was then used to quantitate the voids appearing in the electron micrographs. Once detected, several parameters were measured: the mean area of individual voids (μm²), the total projected area of voids (μm²), the % voids (the total projected area of voids divided by the total area scanned), the total number of voids and the size distributions of the mean areas.

Statistical analysis

The experiment has two factors, each at two levels: (1) location of analyzed region, root versus tip; and (2) treatment, no shampooing versus shampooing. Four independent test conditions were examined with a sample size of 40 each; thus, a two-factor analysis of variance with no interaction model was considered. However, the equal variance assumption was violated and, in order to employ the analysis of variance technique, the four test conditions were combined into one sample and assigned ranks. Since all comparisons involved testing one group mean to another, the Wilcoxon rank-sum test was a plausible test statistic. Because the analysis of variance technique does not apply to the Wilcoxon test, an alternative is provided by Fisher and Yates' normal scores test which transforms the ranks to standard normal values. These values were then analyzed by the analysis of variance technique. It should be noted that there were no gross violations of assumptions using the transformed variates.

All t-values obtained from the analysis of variance techniques were adjusted downward to reflect the Pittman efficiency of the Wilcoxon test to the Student t-test. This adjustment employed the lower bound of V/0.864 as a multiplying factor for all t-values in an effort to be conservative in light of the transformations that were employed.
RESULTS AND DISCUSSION

SUBJECTIVE ANALYSIS

Electron microscope examination of the transverse hair sections showed striking differences between the root and tip ends of the hair fiber. Hair from the tip end of the fiber exhibited discrete holes or voids in the endocuticle region of the cuticle measuring 0.1 to 0.2 mm in diameter. In the cortex, there were numerous voids which appeared to be associated with the intermacrofibrillar matrix, nuclear remnants, and cell membrane complexes. Hair from the root exhibited fewer holes in both cuticle and cortex regions of the fiber.

Whether the observed voids are originally present in the hair fiber as such or represent latent ultrastructural modification which becomes overt during the techniques of sample preparation is not known and is one of the unresolved problems of microscopy. However, these voids are clearly a manifestation of changes occurring in fiber ultrastructure as a direct result of the weathering process.

A subjective analysis of these observations was conducted which consisted of a blind ordinal rating of the photomicrographs for the degree of ultrastructural modification. A scale of 0 to 3 was used, with 0 representing the least modification. After identifying the photomicrographs, the average rating for each treatment group was determined. The root or non-weathered region received an average rating of 0.5 for both non-shampooed and shampooed treatment groups. In other words, no differences were seen which could be related to the multiple shampooing treatment in the root region. In the tip or weathered region, there was a statistically significant increase in the disruption observed compared with the root region. The ratings were 2.4 for the non-shampooed and 2.6 for the shampooed hair. The increase from shampooing, however, was not statistically significant.

Table I
Ultrastructural Changes as a Result of Weathering*

<table>
<thead>
<tr>
<th>Location</th>
<th>% Voids</th>
<th>Total Number of Voids</th>
<th>Mean Area of Voids (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>0.365</td>
<td>33</td>
<td>0.189</td>
</tr>
<tr>
<td>Tip</td>
<td>1.050</td>
<td>76</td>
<td>0.176</td>
</tr>
<tr>
<td>Cuticle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>0.0335</td>
<td>5</td>
<td>0.00710</td>
</tr>
<tr>
<td>Tip</td>
<td>0.412</td>
<td>35</td>
<td>0.0358</td>
</tr>
</tbody>
</table>

* Each value represents the median of 40 measurements.

IMAGE ANALYSIS

Weathering Effects. Statistical analysis of the objective comparisons between root and tip regions of the hair were in agreement with the subjective analysis that was performed. At a greater than 95% confidence level, all measures of ultrastructural disruption, except mean area of cortex voids, increased from the root to the tip (Table I). In the cortex, the mean area of voids showed no difference between tip or weathered hair and root or non-weathered hair. This indicated that the size of the voids which appeared
in the cortex did not change with weathering. The total number of voids in weathered hair was more than double that in non-weathered hair. An even greater increase was made apparent by a measure of the % voids, which is the total area of voids divided by the total area scanned. Therefore, the % voids is the more sensitive measure of ultrastructural change.

In the cuticle region of the hair, ultrastructural changes resulting from the weathering process were more dramatic than those seen in the cortex. The size, number, and % voids all increased in the weathered hair. Although linear voids appearing between cuticle layers were measured, this data was not included in the analysis. These voids appeared to occur randomly within all treatment groups and could not be unambiguously separated from defects originating during sectioning and/or irradiation under the electron beam.

Shampooing Effects. Further analysis of the data to investigate the effect of chronic shampooing on hair fiber ultrastructure suggested an interactive effect between shampooing and location on the hair fiber (Tables II and III). In the root or non-weathered region of the hair fiber, no ultrastructural differences were noted between shampooed or non-shampooed fibers. Similarly, shampooing was found to have no effect in the cortex region of weathered hair fibers: the mean area of voids, total number of voids, and % voids were essentially unaffected by the shampooing treatment. However, in the cuticle of weathered hair, the mean area of voids, total number of voids, and % voids were greater in shampooed hair fibers than in non-shampooed fibers. The increase in the % voids measurement in shampooed hair was statistically significant at the greater than 95% confidence level.

The ultrastructural impact of shampooing is, therefore, greater in tip or weathered regions of the hair than in root or non-weathered regions. In addition, only the cuticle or outer portion of the hair was shown to be affected by the multiple shampooing treatment. Thus, the trend that appeared to be taking place in the subjective analysis has been verified using the image analysis technique.

CONCLUSION

The current study demonstrates that electron microscopy used in conjunction with image analysis can be used to quantitatively assess subtle environmental and cosmetic effects on the hair fiber. Using this technique, we have found that the cumulative process of

<table>
<thead>
<tr>
<th>Table II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrastructural Changes in Non-Weathered Hair as a Result of Shampooing*</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Cortex</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Cuticle</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* Each value represents the median of 40 measurements.
Ultrastructural Changes in Weathered Hair as a Result of Shampooing*  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Voids</th>
<th>Total Number of Voids</th>
<th>Mean Area of Voids (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>Non-Shampooed</td>
<td>1.05</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Shampooed</td>
<td>0.903</td>
<td>67</td>
</tr>
<tr>
<td>Cuticle</td>
<td>Non-Shampooed</td>
<td>0.412</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Shampooed</td>
<td>0.725</td>
<td>57</td>
</tr>
</tbody>
</table>

* Each value represents the median of 40 measurements.

Weathering is reflected in ultrastructural changes in the cuticle and cortex of hair fiber cross sections. In addition, the impact of chronic shampooing on hair fiber ultrastructure is limited to the cuticle region of weathered hair fibers.

The use of electron microscopy to assess subtle changes in fiber structure requires that a representative sampling be made and a sufficiently large set of micrographs be reviewed. Image analysis allows a more objective quantitative approach to electron micrograph interpretation and allows one to analytically verify observed ultrastructural effects.

ACKNOWLEDGEMENTS

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REFERENCES