

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/275661222>

Advanced Hair Damage Model from Ultra-Violet Radiation in the Presence of Copper

Article in *International journal of cosmetic science* · April 2015

DOI: 10.1111/ics.12231 · Source: PubMed

CITATIONS

2

READS

61

11 authors, including:



Michael G Davis

Procter & Gamble

29 PUBLICATIONS 1,310 CITATIONS

[SEE PROFILE](#)



Yiping Sun

Procter & Gamble

35 PUBLICATIONS 1,200 CITATIONS

[SEE PROFILE](#)



Tanuja Chaudhary

Procter & Gamble

15 PUBLICATIONS 692 CITATIONS

[SEE PROFILE](#)



Laia Rubio

Instituto de Química Avanzada

36 PUBLICATIONS 250 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



3D titanium [View project](#)



Impact of additives on microstructures in vesicular suspensions [View project](#)

All content following this page was uploaded by [L. Coderch](#) on 26 August 2015.

The user has requested enhancement of the downloaded file. All in-text references [underlined in blue](#) are added to the original document and are linked to publications on ResearchGate, letting you access and read them immediately.

Advanced hair damage model from ultra-violet radiation in the presence of copper

J. M. Marsh*, M. G. Davis*, M. J. Flagler*, Y. Sun*, T. Chaudhary*, M. Mamak*, D. W. McComb†, R. E. A. Williams†, K. D. Greis‡, L. Rubio§ and L. Coderch§

*The Procter and Gamble Company, Mason Business Center, 8700 Mason Montgomery Road, Mason, OH 45040, U.S.A. †Center for Electron Microscopy and Analysis, The Ohio State University, 1305 Kinnear Road, Columbus, OH 43212, U.S.A. ‡Proteomics and Mass Spectrometry Laboratory, University of Cincinnati College of Medicine, 3125 Eden Ave., Cincinnati, OH 45267, U.S.A. and §IQAC-CSIC, C/Jordi Girona 18-26, 08034 Barcelona, Spain

Received 3 February 2015, Accepted 25 March 2015

Keywords: chelants, copper, free radicals, hair, histidine, mass spectrometry, N,N' ethylenediamine disuccinic acid, transmission electron microscopy, UV X-ray energy dispersive spectroscopy

Synopsis

OBJECTIVE: Damage to hair from UV exposure has been well reported in the literature and is known to be a highly complex process involving initiation via absorption of UV light followed by formation and propagation of reactive oxygen species (ROS). The objective of this work was to understand these mechanisms, explain the role of copper in accelerating the formation of ROS and identify strategies to reduce the hair damage caused by these reactive species.

METHODS: The location of copper in hair was measured by Transmission electron microscopy–(TEM) X-ray energy dispersive spectroscopy (XEDS) and levels measured by ICP-OES. Protein changes were measured as total protein loss via the Lowry assay, and MALDI ToF was used to identify the biomarker protein fragments. TBARS assay was used to measure lipid peroxide formation. Sensory methods and dry combing friction were used to measure hair damage due to copper and UV exposure and to demonstrate the efficacy of N,N' ethylenediamine disuccinic acid (EDDS) and histidine chelants to reduce this damage.

RESULTS: In this work, a biomarker protein fragment formed during UV exposure is identified using mass spectrometry. This fragment originates from the calcium-binding protein S100A3. Also shown is the accelerated formation of this peptide fragment in hair containing low levels of copper absorbed from hair during washing with tap water containing copper ions. Transmission electron microscopy (TEM) X-ray energy dispersive spectroscopy (XEDS) studies indicate copper is located in the sulphur-poor endo-cuticle region, a region where the S100A3 protein is concentrated. A mechanism for formation of this peptide fragment is proposed in addition to the possible role of lipids in UV oxidation. A shampoo and conditioner containing chelants (EDDS in shampoo and histidine in conditioner) is shown to reduce copper uptake from tap water and reduce protein loss and formation of S100A3 protein fragment. In addition, the long-term consequences of UV oxidation and additional damage induced by copper are illustrated in a four-month wear study where hair was treated with a consumer rele-

vant protocol of hair colouring treatments, UV exposure and regular shampoo and conditioning.

CONCLUSIONS: The role of copper in accelerating UV damage to hair has been demonstrated as well as the ability of chelants such as EDDS and histidine in shampoo and conditioner products to reduce this damage.

Résumé

OBJECTIF: Les dommages aux cheveux de l'exposition aux UV sont bien décrits dans la littérature et sont connus pour être un processus très complexe impliquant l'initiation par absorption de la lumière UV suivie par la formation et la propagation des espèces réactives de l'oxygène (ROS). L'objectif de ce travail était de comprendre ces mécanismes, d'expliquer le rôle du cuivre dans l'accélération de la formation de ROS et d'identifier des stratégies pour réduire les dommages aux cheveux causés par ces espèces réactives.

MÉTHODES: L'emplacement du cuivre dans les cheveux a été mesuré par spectroscopie d'énergie de rayons X à dispersion en microscopie électronique de transmission (TEM) (XEDS) et les niveaux quantitatifs ont été mesurés par ICP-OES. Les modifications de protéines ont été mesurées comme la perte de protéine totale par le dosage de Lowry, et MALDI ToF a été utilisé pour identifier les fragments de protéines des biomarqueurs. Le dosage TBARS a été utilisé pour mesurer la formation des peroxydes lipidiques. Des méthodes sensorielles et la friction de peignage à sec ont été utilisées pour mesurer les dommages de cheveux dus au cuivre et à l'exposition aux UV et de démontrer l'efficacité d'EDDS et des chélateurs à l'histidine à réduire ces dommages.

RÉSULTATS: Dans ce travail, un fragment d'une protéine biomarqueur formé au cours de l'exposition aux UV est identifié par spectrométrie de masse. Ce fragment provient de la protéine S100A3 fixant le calcium. On voit également la formation accélérée de ce fragment peptidique dans les cheveux contenant de faibles niveaux de cuivre absorbé pendant le lavage des cheveux avec des ions de cuivre contenus dans l'eau du robinet. La Microscopie électronique en transmission (TEM) et la spectroscopie à l'énergie de rayons X dispersive (XEDS) indiquent que le cuivre est situé dans la région endo-cuticulaire pauvre en soufre; une région où la protéine S100A3 est concentrée. Un mécanisme de formation de ce

Correspondence: Jennifer Marsh, The Procter and Gamble Company, Mason Business Center, 8700 Mason Montgomery Road, Mason, Ohio 45040, U.S.A. Tel.: +1 513 622 0445; fax: +1 513 386 2533; e-mail: marsh.jm@pg.com

fragment peptidique est proposé en plus du rôle possible des lipides dans l'oxydation UV. Un shampooing et un conditionneur contenant des chélateurs (EDDS en shampooing et histidine dans le conditionneur) sont indiqués pour réduire l'absorption de cuivre de l'eau du robinet, réduire la perte de protéines et la formation du fragment de protéine S100A3. En outre, les conséquences à long terme de l'oxydation UV et des dégâts supplémentaires induites par le cuivre sont illustrés dans une étude d'usure de quatre mois où les cheveux ont été traités avec un protocole pertinent, simulant les soins de coloration des cheveux, l'exposition aux UV et les shampooing et conditionnement habituels.

CONCLUSIONS: Le rôle du cuivre dans l'accélération de dommages des rayons UV sur les cheveux a été démontré ainsi que la capacité des chélateurs tels que EDDS et histidine dans des produits shampooing et conditionneur de réduire ces dommages.

Introduction

Damage to hair from exposure to UV and visible light has been well documented in the literature [1,2]. It has been shown that this damage can occur to the keratin proteins [3], lipids [4] and melanin [5] eventually leading to colour changes [6] and also noticeable physical changes such as split ends, loss of shine and manageability. Maintaining hair health is a key need for women globally, so understanding the mechanisms of UV damage and identifying technology to prevent this damage is a high priority for the cosmetics industry.

A hair harvest study conducted globally demonstrated that copper is present in women's hair at levels between 10 and 200 $\mu\text{g g}^{-1}$ and that the majority of this copper comes from impurities in tap water [7]. In a previous publication [8], it was shown that the presence of this copper in hair, even at low levels, can accelerate UV damage and several mechanisms were proposed involving the production of reactive oxygen species (ROS). In this study, a peptide biomarker (MW 1277.7) for UV damage was detected by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry in both hair water extracts and directly on hair fibre surfaces. Its concentration in water extracts was determined to be related to both the level of UV exposure and the copper level in hair. Further, mass spectrometry sequencing of the peptide marker observed here showed it is a fragment of S100A3 protein. This protein has been previously reported to be involved in cuticle cell adhesion [9] and been determined to be located primarily in the endocuticle and to a lesser extent in the cortex [10]. Scanning transmission electron microscopy-X-ray energy dispersive spectroscopy (STEM-XEDS) studies have shown that exogenous copper in hair from tap water is located in the endo-cuticle and a mechanism is proposed for formation of the biomarker (MW 1277.7) in the presence of copper based on this finding. Copper has also been shown to participate in lipid oxidation mechanisms, proposed to be occurring in the cuticle cell membrane complex. In addition, it has been shown that chelation of copper via a regimen of chelants in shampoo and conditioner can significantly reduce UV damage to hair and ultimately lead to improved hair health.

Materials and methods

Hair source

Chemically virgin Caucasian-source hair was purchased from International Hair Importers & Products Inc. (Glendale, NY, U.S.A.).

Individual tresses (2 g, 6in flat with hot wax tab at top), formed by evenly blending hair from multiple ponytails, were used for all experiments. Coloured hair was created by treating virgin hair tresses once with an oxidative permanent commercial colourant (Nice 'n' Easy 98 Extra Light Blonde Shade). The mixed colourant was thoroughly massaged onto hair at a dose of 4 g of product per gram of hair then incubated for 30 min in an oven at 30°C. The product was then completely rinsed from hair.

Protein loss measurements

0.2–0.3 g hair samples (2 in. length) were collected from each hair tress and were added to glass scintillation vials. Distilled water was added at a ratio of 10 : 1 (mL water to g hair). Samples were shaken for 1 h at 2500 rpm on a DVX-2500 Multitube Vortexer platform (VWR International, Radnor, PA, U.S.A.). Following direct measurement of total protein, samples were subjected to centrifugation at 16 100 *g* to separate into soluble/insoluble fractions. Pelleted material (insoluble) was solubilized in 3 M urea, 1 M NaOH, 0.06% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) followed by sonication for 30 min in a Branson B300 sonicating water bath (34 kHz). Protein concentration in soluble/insoluble fractions was determined using the Modified Lowry assay against a porcine gelatin standard (Modified Lowry Protein Assay kit supplied by Pierce, Rockford, IL, <http://www.pierce-net.com>).

MALDI-TOF mass spectrometric analyses of water extracts of UV damaged hair and MALDI-imaging of damage biomarkers directly on single hair fibres

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used for fast detection of peptide marker ions specific to UV damaged hair. Briefly, 5 μL of each water extract from virgin and LLS hair was mixed with 5 μL of MALDI matrix α -cyano-4-hydroxycinnamic acid (CHCA at 10 mg mL^{-1} in 80% Acetonitrile/Water/0.1% trifluoroacetic acid). 0.7 μL of this mixture was spotted on a target plate and allowed to air dry at room temperature before MALDI analysis. A MALDI TOF/TOF 4800 Plus Mass Analyzer (AB-Sciex, Framingham, MA, U.S.A.) was used in the positive ion reflectron mode. The mass spectrometer uses a 200-Hz frequency Nd:YAG laser, operating at a wavelength of 355 nm. Ions generated by the MALDI ionization process were accelerated at 20 kV. MALDI-TOF mass spectra were typically generated in the mass range 800–4000 Da. Data were collected in an automated fashion using random sampling over the sample spot with 50 shots per subspectrum and a total of 1000 shots per spectrum. The intensity of peptide marker peaks for each extract was measured. For the peptide biomarker sequencing, hair water extracts were analysed by online NanoLC (Waters, NanoAcquity, Milford, MA, U.S.A.) high-resolution Orbitrap Elite mass spectrometry (Thermo Fisher, Schaumburg, IL, U.S.A.). The NanoLC used 5–60%B over 60 min with a 75 $\mu\text{m} \times 5 \text{ cm}$ C18 column and Easyspray interface (Thermo Fisher). The LC solvent A was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The Orbitrap system provides mass measurement accuracy better than 10 ppm. Mascot software (Matrix Science Corporation, London, U.K.) was used to search Swiss-Prot protein database (Swiss Institute of Bioinformatics, Switzerland) to identify the peptide sequence of the biomarker. Protein database search of the NanoLC-Orbitrap data was done with some common modifications,

for example N-terminal acetylation, methionine oxidation, amidation, etc.

MALDI-Imaging mass spectrometry was used to directly detect and visualize the peptide markers from damages on hair fibres. The marker peptide intensity in a MALDI image was acquired in the same data acquisition for comparison. In this study, single hair fibres (~6 cm in length) from virgin, bleach and UV exposure were mounted directly on a MALDI plate using a piece of double-sided, conductive adhesive tape. After spraying with the MALDI matrix α -cyano-4-hydroxycinnamic acid (CHCA, 10 mg mL⁻¹ 80%ACN/H₂O/0.1%TFA), the hair fibre surface was air-dried and analysed in the imaging mode by rastering the laser beam across the area (200 μ m rastering, laser intensity 4500, mass scanning range of 800–1400 Da, 250 shots per spectrum). The MALDI-imaging data were processed using Biomap imaging software developed by Markus Stöckli (Novartis, Basel, Switzerland). The ion intensity map on a specific peptide marker, for example m/z 1278, was generated for a comparison of virgin, bleached and UV damage hair conditions.

Determination of metal content in hair

Metal content of hair samples was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) with an Optima 5300 DV Optical Emission Spectrometer (Perkin Elmer Life and Analytical Sciences, Shelton, CT, U.S.A.). Samples of 100 mg of hair were digested overnight with 2 mL of high purity concentrated nitric acid. The digestive mixture also contained 150 μ L of 100 μ g g⁻¹ yttrium internal standards (Inorganic Ventures, Christiansburg, VA, U.S.A.). Following digestion, samples were heated to 70–80°C for 1 h, cooled to room temperature and diluted to 15 mL with deionized water. Each hair sample was analysed in triplicate.

Exposure to artificial radiation

Sun exposure was simulated by irradiation with an Atlas Ci3000+ weather-o-meter (Atlas, Chicago, IL, U.S.A.). An internal and outer quartz filter was used to simulate broad-spectrum, outdoor daylight with a specific irradiance of 1.48 W m⁻² at 420 nm. During the irradiation process, temperature and relative humidity (RH) were kept constant at 35°C and 80% RH.

STEM-XEDS copper mapping in hair cross sections

Cross-sectional hair samples for scanning transmission electron microscopy (STEM) analysis were prepared using a dual-beam focused ion beam (FIB) instrument (FEI Helios). The hair sample was coated with Au using a Leica ACE600 (Leica Microsystems Inc, Buffalo Grove, Illinois, USA) prior to sectioning to increase conductivity and also act as a protective layer against unwanted ion sputtering. Cross-sectional lamellae were extracted using an Omniprobe Autoprobe™ 200 and placed on molybdenum Omniprobe TEM grid for final thinning. Samples were thinned finally to electron transparency at 30 kV, 93pA [11].

(S)TEM-XEDS was performed using a FEI Titan™ G2 60–300 kV (S)-TEM equipped with the 4-quadrant, silicon drift detector Super-X XEDS system and X-FEG high brightness source. Spectral imaging (SI) maps were collected in a 1024 \times 1024 array with a 20 μ s dwell/pixel applying an iterative drift correction between frames to sum spectral data [12]. Total acquisition time for SI maps was 20 min allowing for post-processing to create line profiles across given regions of interest. Electron beam probe size was ~1.5 Å.

Lipid peroxide measurements

To evaluate lipoperoxidation, a thiobarbituric acid (TBARS) assay was performed [13]. It is based on the reaction of malondialdehyde (MDA) (one of the products formed by the decomposition of lipoperoxidized compounds) with thiobarbituric acid (TBA) to form a coloured complex (MDA:TBA).

About 500 mg of the different hair samples was weighted. Then, lipids from each of the different hair sample were extracted with methanol (500 mg hair per 10 mL methanol) in a sonicated device Labsonic 1510 (B. Braun, Melsungen, Germany) for 15 min (room temperature). Next, the hair extracts were dried under a N₂ current and diluted again in 1.5 mL of methanol.

The results are expressed as malonaldehyde bis(dimethylacetal) equivalents (μ M MDA) using a standard curve for pure MDA–TBA complex. The calibration curve was obtained by using MDA at different concentrations (0.5–30 μ M). The absorbance values obtained from hair samples allow to find the amount of peroxidated lipid of each sample. For this, the absorbance values from each of the samples are extrapolated in the equation of the calibration curve obtained, giving results expressed in μ M equivalent to MDA peroxides formed. As final results, MDA equivalents found relates with the initial amount of hair obtaining the amount of peroxides formed for each mg of hair.

Chelant testing

N,N' ethylenediamine disuccinic acid (EDDS) at 0.1% active level was added to the test shampoo, Pantene Volume, and histidine at 0.05% active level was added to the test conditioner, Pantene Volume. Virgin hair was washed for 30 cycles with shampoo plus conditioner and then analysed for copper uptake using the ICP method. Each wash cycle consisted of applying 0.1 g g⁻¹ shampoo to the hair tress and lathering for 30 s followed by a 30-s rinse repeated for a total of two shampoo applications. Conditioner was then applied at 0.1 g g⁻¹ and thoroughly distributed along the tress for 30 s followed by a 30-s rinse. Hair was then dried in a hot box at 80°C. Three tresses per leg were tested.

Wear protocol and testing

Frizzy hair (4 g 15 cm tresses) that had been treated with a hair colourant was used for the study. N,N' ethylenediamine disuccinic acid at 0.1% active level was added to the test shampoo, Pantene Aqualight and histidine at 0.1% level was added to the test conditioner, Pantene Aqualight. The hair was first washed for 45 cycles using the protocol above with shampoo plus conditioner. It was then treated with a hair colourant (Koleston shade 10/0) by applying 4 g g⁻¹ of colourant to the tress and leaving for 30 mins in an oven at 30°C before rinsing thoroughly. Following treatment with the colourant, a 6-week wear protocol was completed where each week consisted of six cycles of shampoo plus conditioner and 3 h of UV exposure (broad-spectrum, outdoor daylight with a specific irradiance of 1.48 W m⁻² at 420 nm). This process was repeated for a total of three times. The wash water was medium water hardness (~170 μ g g⁻¹ calcium carbonate) and 0.05 μ g g⁻¹ copper ions. Five tresses per leg were treated.

Dry combability was measured by an Instron Model 5542 Tensile Tester (Instron Industrial Products, Grove City, PA, U.S.A.)

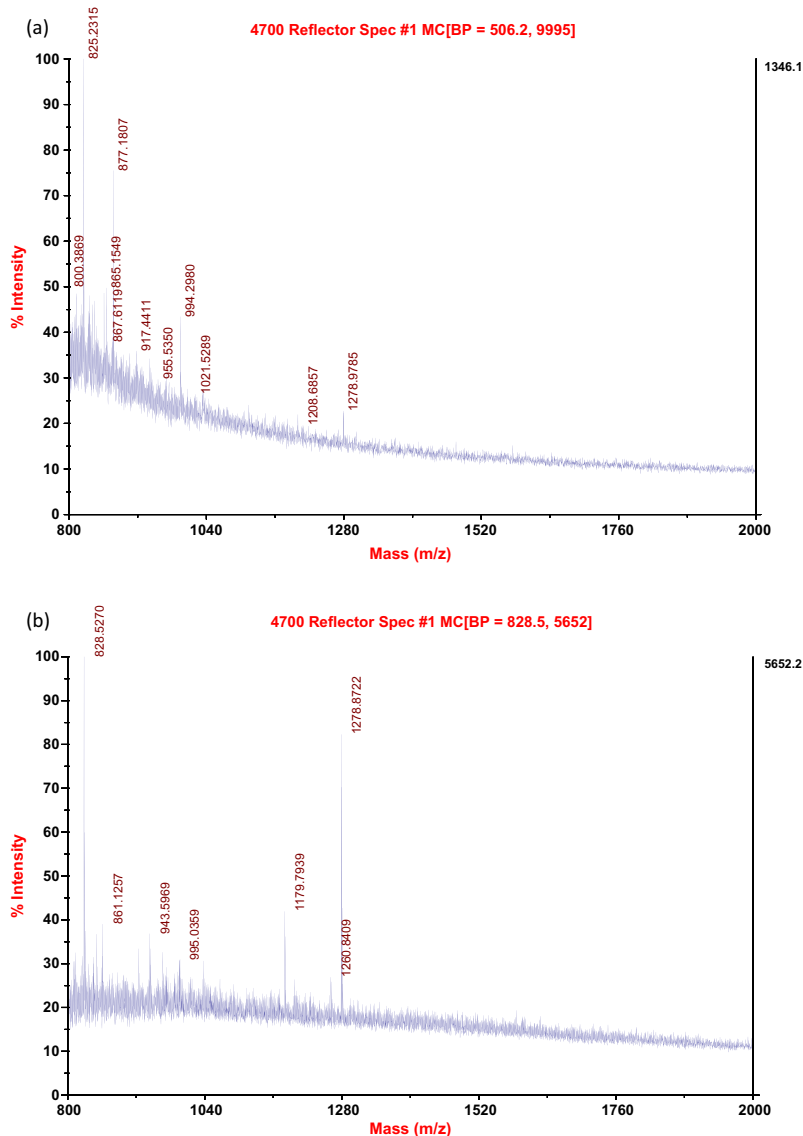


Figure 1 Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry spectra for (a) untreated hair and (b) hair exposure to 40 h UV/visible light.

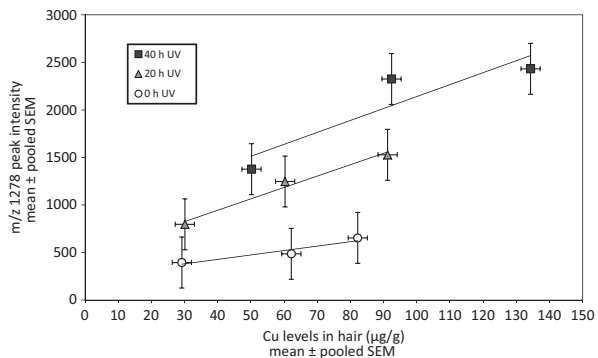


Figure 2 $m/z = 1278.7$ biomarker concentration as UV and copper level in hair is increased.

equipped with a 50 N load cell and a set of fine-toothed combs. Tresses ($n = 3$) were mounted onto the load cell and combed five times by the instrument. Dry combability was characterized by end peak forces experienced by the top comb (comb 2).

Results and discussion

Identification of UV biomarker

The protein fragments eluted from treated hair shaken in water for an hour were analysed using matrix-assisted laser desorption ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry (Fig. 1). A peak was observed at $m/z 1278.7$, which was consistently seen for hair exposed to UV/visible light. Figure 2 shows this biomarker concentration as UV exposure is increased as copper in hair is increased

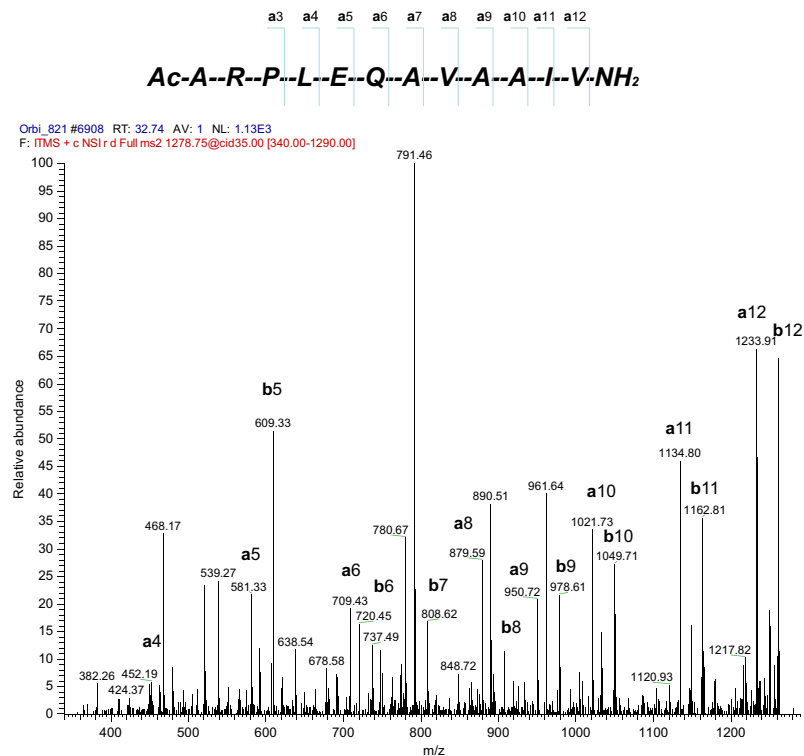


Figure 3 MS/MS peptide sequencing of the m/z 1278.7 biomarker using NanoLC-high-resolution Orbitrap identifies S100A3 as the parent protein and indicating that this specific fragmentation of S100A3 is a unique biomarker for UV-stimulated hair damage. The sequence of this marker fragment is Ac-AR-PLAQVAAIV-NH₂ (MW 1277.7457). Fragment ions are mainly from a and b series ions.

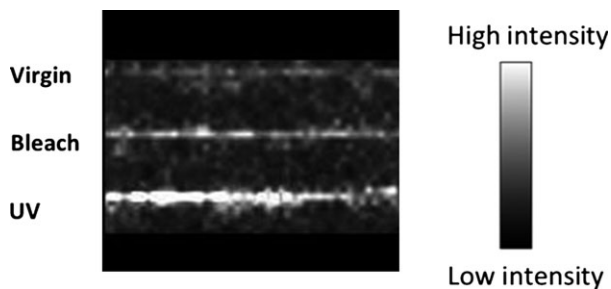


Figure 4 MALDI Image of virgin (top), bleached (middle) and UV-exposed (bottom) hair fibres. The UV damage peptide marker (m/z 1278, a fragment of S100A3) was detected and mapped directly on hair surfaces. The hair exposed to 40 h UV showed intense signal than those from both virgin and bleached hair.

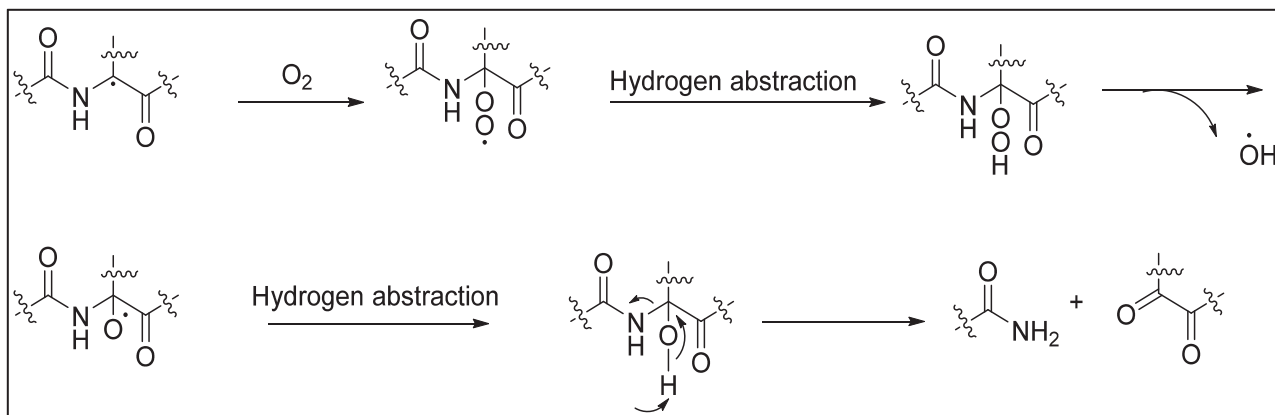
clearly demonstrating the correlation between UV exposure, copper level and formation of this peptide fragment m/z 1278.7.

Figure 3 demonstrates the MS/MS peptide sequencing of the m/z 1278.7 biomarker using NanoLC-high-resolution Orbitrap, which identified S100A3 as the parent protein. The UV damage marker ion m/z 1278.7 matched the sequence Ac-AR-PLAQVAAIV-NH₂ (Molecular Weight = 1277.7457), with 0.1 ppm accuracy to the calculated value (1277.7455), therefore an accurate sequence determination. The mass spectrometric fragment ions from the marker m/z 1278.7 showed mainly a series ions (e.g., 452.2, 581.3, 709.4, 780.7, etc.) and b series ions (e.g., 609.3, 737.4, 1049.7, 1162.8, etc.) due to the arginine residue located near the

N-terminal of this peptide. In addition to the singly charged ion m/z 1278.7, the doubly charged ion m/z 640.3 of this marker peptide is also detected in the same nanoLC-Orbitrap analysis, with a mass measurement accuracy of -0.07 ppm. Mascot software is used to search Swiss-Prot protein database to identify that the parent protein of the marker is S100A3. The complete sequence of this protein is MARPLEQAVAAIVCTFQEYAGRCGDKYKLCQAEIKELKQKELATWTPTEFRECDYNKFMVSLDTNKCDEVDFVEYVRSALACLCLYCHEYFKDCPSEPPCSQ with the underlined section the biomarker fragment eluted into water after UV exposure. It has been acetylated at the arginine end and an amide formed at the valine end of the peptide.

In addition to the detection of the specific UV damage peptide marker m/z 1278.7 in water extracts of hair using MALDI and nanoLC-Orbitrap mass spectrometry, MALDI mass spectrometry imaging (MALDI-Imaging) was used to detect and map the distribution directly on hair surface. Figure 4 is the MALDI image from an imaging area of 2 cm along the hair fibres. The peak intensity of m/z 1278 from hair exposed to UV (bottom) was much more intense than the virgin (top) and bleached (middle) hair. This is consistent to the MALDI result from the water extracts of the hair.

The S100A3 protein has been well studied in various substrates including hair. It is part of the S100 family of proteins that contain 2 EF-hand calcium-binding motifs and are localized in the cytoplasm and/or nucleus of a wide range of cells. The S100A3 protein in hair has been shown to strongly bind both calcium and zinc and to be localized in the endo-cuticle with some expression in the cortex matrix [14]. Inoue and Kizawa have hypothesized that it is important for cuticle-cuticle cell adhesion [15] and it has also been demonstrated that it can be liberated by chemical treatments. PMOL software (DeLano Scientific, Portland, Oregon, USA) was used to



Scheme 1 Possible pathways for breakdown of S100A3 protein to form biomarker peptide.

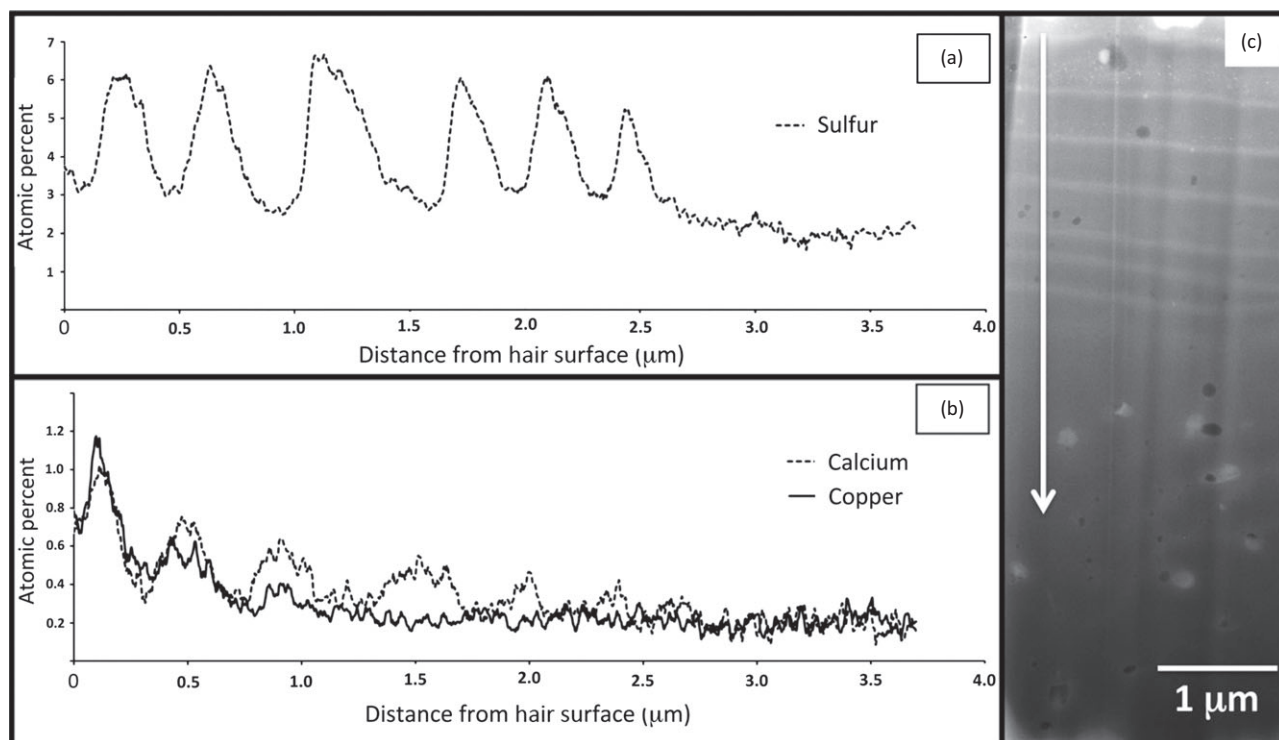
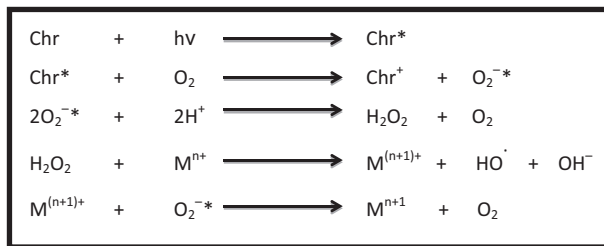


Figure 5 Plots showing the variation in atomic percent, as measured by XEDS, of (a) sulphur K-alpha and (b) copper K-alpha along the direction indicated by the white arrow in the (c) HAADF-STEM image of the cross-section of the hair.

determine the location of the peptide in the three-dimensional structure of S100A3 (PDB ID: 3NSO) (Unno *et al.* 2011 JMB), which showed that a modelled binding site for zinc is close to the valine–cysteine peptide linkage which is broken to form the biomarker fragment. It is highly probable that the copper from tap water will exchange with calcium at this binding site and thus form a highly reactive radical species on exposure to UV that preferentially forms this fragment. Scheme 1 shows a possible pathway to break the protein chain in the presence of a reactive radical.

Identification of copper location

STEM-XEDS was used to identify the location of copper in hair introduced via tap water. The dual-beam FIB was used to create ~100 nm sections required for analysis [1]. For direct, spatial characterization of copper in hair, the dual-beam FIB provided a specific and clear advantage over traditional embedding/ultramicrotomy approaches as it removed the opportunity for species to migrate during the optimal sample preparation process. During this work,



Scheme 2 Proposed pathway for metal-induced radical formation.

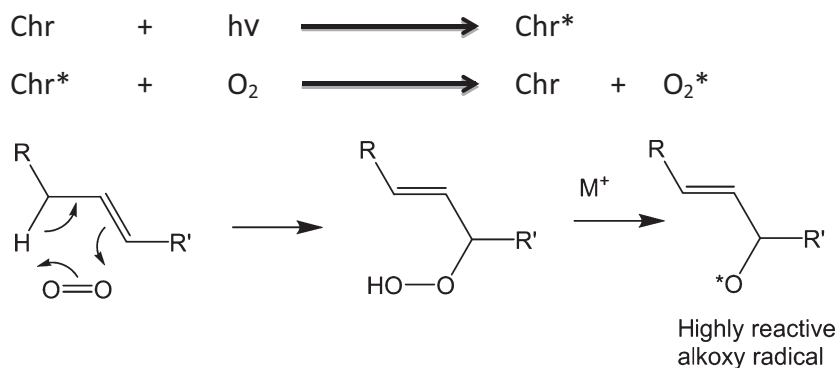
ultra-thin sections were also prepared by conventional ultramicrotomy methods, that is mounting hair in resin, sectioning with a diamond knife, floating sections onto water, and then lifting onto a gold TEM grid with support film. When analysed by STEM-XEDS, these specimens showed *no* significant copper signal above the background signal. It is proposed that copper migrated from the hair during the resin incorporation step or by dissolution as the section was briefly floated onto water.

Figure 5 shows a XEDS line scan from the outer edge of hair through the cuticle cells to $\sim 4 \mu\text{m}$ depth. The sulphur K_α signal varies with an expected periodicity correlating to higher levels in the sulphur-rich exo-cuticle and A-Layer and lower levels in the sulphur-poor endo-cuticle. Profiles of the copper and calcium K_α signals show clearly the presence of these metallic species in the cuticle and concentrated in sulphur-poor areas of the cuticle, that is the endo-cuticle. The Cu profile in Fig. 5 also indicates copper is concentrated in the outer three or four cuticle layers.

The location of copper in the endo-cuticle fits well with the proposed mechanism for formation of the $m/z = 1278.7$ biomarker fragment with copper forming a reactive radical where it is bound to the S100A3 protein. Scheme 2 shows a possible route to form this reactive radical where radical pathway initiation comes from absorption of UV light by chromophores in hair such as amino acid residues tryptophan and tyrosine. As this mechanism involves tryptophan as a chromophore in hair that absorbs UV, we may expect to see a difference in tryptophan fluorescence with and without copper in hair.

Lipid oxidation

Hoting and Zimmerman [4] have shown changes to hair lipids on exposure to UV light and they proposed that oxidation occurs



Scheme 3 Proposed pathway for UV oxidation of unsaturated lipids in hair.

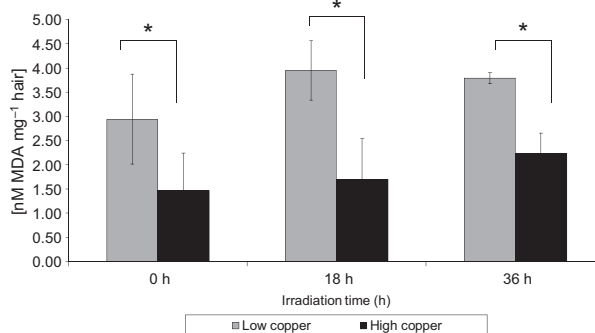


Figure 6 Lipid peroxide formation after UV exposure (* = >95% significance).

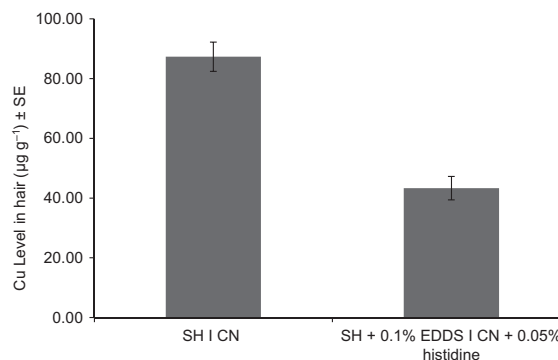


Figure 7 Copper level in hair after 20 wash cycles.

primarily to unsaturated lipids such as unsaturated fatty acids and wax esters. It has been shown by several groups that the internal lipids in hair found in the cuticle and cortex cell membrane complex contain high levels of fatty acids including unsaturated fatty acids such as oleic acid and palmitoleic acid [16]. Scheme 3 shows a proposed mechanism where singlet oxygen is formed via UV absorption by chromophores in hair such as tryptophan and tyrosine. The singlet oxygen can react with the double bond of the unsaturated lipid via an 'ene' reaction to form a lipid peroxide. In the presence of redox metals such as copper, an alkoxy radical is

formed. This radical is highly reactive radical and will propagate additional radical reactions that will further break down both lipid and protein structures of hair.

Hair with high and low copper levels was exposed to UV irradiation and then the internal lipids were extracted from hair using methanol, and then the lipid peroxides measured via the thiobarbituric acid (TBARS) assay. This assay is based on the reaction of malondialdehyde (MDA) (one of the products formed by the decomposition of lipid-peroxidized compounds) with thiobarbituric acid (TBA) to form a coloured complex (MDA:TBA). Figure 6 shows the lipid peroxide data for hair exposed to 0, 18 and 36 h of UV with a base level of copper ($17 \mu\text{g g}^{-1}$) and a higher level ($78 \mu\text{g g}^{-1}$). These data support the proposed Scheme 3 where the presence of copper reduces the overall level of lipid peroxides in hair by forming reactive radicals. Also noted is that even at 0 hrs of UV exposure, there are lipid peroxide present in the hair tested. This is may not be surprising as the hair used has previously been exposed to UV before it was harvested. Addition of copper to this substrate can thus initiate radical reactions and hair damage even without further UV exposure.

Removal of copper from hair

Previously published work showed that addition of a chelant such as N,N'-ethylenediamine disuccinic acid can reduce the level of copper in hair when it is added to a shampoo [8]. An attempt to formulate this chelant into a conditioner was unfortunately not possible due to the chelant's high negative charge, which led to instability of the conditioner's gel network structure. It was determined that histidine could be formulated into a conditioner at levels up to 0.5% and that this active showed a high efficacy to remove copper from hair. The identification of histidine as an efficient copper chelant was not an obvious solution, but it was shown to be superior in performance when compared to other more traditional chelants such as ethylenediamine tetracetic acid (EDTA) and

1-hydroxyethane 1,1-diphosphonic acid (HEDP). It was also superior when compared to other amino acids such as arginine, glycine, proline and alanine. An important factor in effectively chelating copper from hair is the ability of the chelant to complex low levels of copper in the presence of high levels of calcium. Data from hair harvesting show copper levels in the range $20\text{--}200 \mu\text{g g}^{-1}$ and calcium levels in the range $450\text{--}13\,000 \mu\text{g g}^{-1}$, and thus, the conditional formation constant for the copper–chelant complex needs to be significantly higher than the calcium–chelant complex. Histidine forms a strong complex with copper due to additional binding via the imidazole group [17], and it has a conditional formation constant for copper at shampoo and conditioner pH that is approximately one hundred times higher than the other amino acids ($\text{Log } K_{\text{cond}} = 10.2$ for histidine).

Figure 7 shows the copper build-up over 20 cycles for hair washed in tap water containing ~ 0.6 ppm copper. The data clearly show the benefit of washing with a shampoo containing EDDS plus a conditioner containing histidine in reducing the final copper level in hair by $\sim 50\%$. This hair was exposed to 40 h of UV (outdoor daylight with a specific irradiance of 1.48 W m^{-2} at 420 nm) and the protein loss and formation of the m/z 1278.7 biomarker measured. Figure 8 shows the data from this study and demonstrates how reducing copper levels in hair via chelants will significantly reduce protein degradation and specifically fragmentation of the S100A3 protein.

In a separate protocol, the chronic benefits of reducing copper levels in hair were explored using a four-month wear study which combined oxidative insults with physical damage from combing, washing, etc. The protocol was designed to mimic a consumer's habits and practices to enable measurement of the combination of insults leading to hair damage. It has been shown from the previous data that hair containing copper when exposed to UV and/or colouring damage will accelerate formation of damaging free radicals which break down both proteins and lipids. This breakdown of the hair structure makes it more susceptible to physical damage

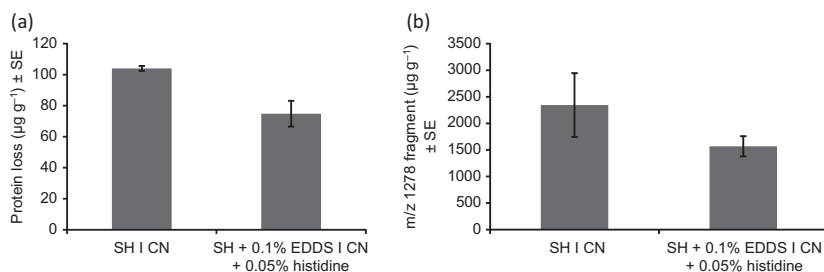
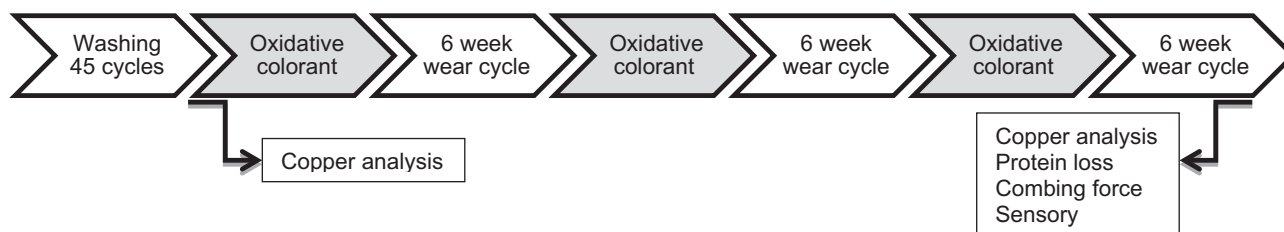


Figure 8 (a) Protein loss, (b) m/z 1278.7 fragment concentration after 20 cycles.



Scheme 4 Wear protocol.

from washing and combing ultimately leading to breakage, loss of shine, poor feel, etc. These are end points, which are indicative to the consumer of poor hair health.

Scheme 4 shows the protocol used in the study, which contained three treatments with an oxidative colourant and 6 weeks

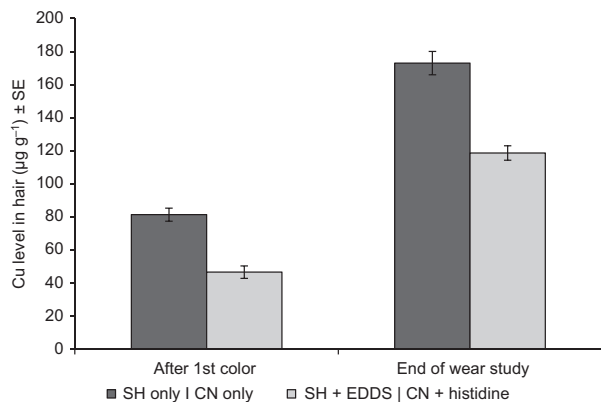


Figure 9 Copper levels in hair after first colour and end of wear.

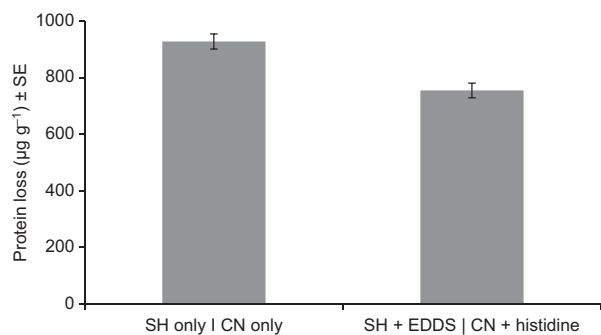


Figure 10 Protein Loss data after wear protocol.

of wear. Each week of wear consisted of six cycles of shampoo and conditioner and 3 h of UV exposure (outdoor daylight, irradiance of 1.48 W m⁻² at 420 nm). The hair (4 g, 15 cm tresses) used was wavy and previously treated with an oxidative hair colourant. The water used for washing was medium water hardness and contained ~0.05 µg g⁻¹ copper ions. Two legs were tested; the first was a shampoo and conditioner with no added chelant and the second was a shampoo with 0.1% EDDS and a conditioner with 0.1% histidine.

Figure 9 shows copper levels in hair before the first oxidative colourant treatment and at the end of the wear protocol. The data show an increased uptake of copper in hair as the wear study progresses and also confirms the ability of chelants in the shampoo and conditioner to reduce copper levels significantly.

The protein loss data at end of the wear protocol (Fig. 10) show reduced protein loss for the shampoo and conditioner with chelants and again this is predicted from previous data where increased copper levels lead to increased free radical formation, which breakdown the cortex and cuticle proteins. At least part of this protein loss will include the *m/z* = 1278.7 fragment of S100A3 protein.

The consumer noticeable consequences of this increased protein loss is illustrated by sensory evaluations by a naïve consumer panel after the wear protocol (Fig. 11a). Each panellist was asked to rate shine of each tress (1 = low shine, 5 = high shine), force required to comb through each tress (1 = poor combing, 5 = good combing) and feel of each tress (1 = poor feel, 5 = good feel). Finally, they were asked to rate overall health of each tress (1 = poor hair health, 5 = good hair health), but no guidance was given as to how to make this assessment. The data show significantly lower scores for all these parameters for hair treated with shampoo and conditioner with no chelant including lower hair health. Figure 11b confirms the sensory findings for combing by measuring combing force for each tress. Hair treated with shampoo and conditioner with chelant has a significantly lower combing force.

Conclusions

Exposure to UV will damage hair as measured by protein loss and this damage will be accelerated in the presence of copper ions from

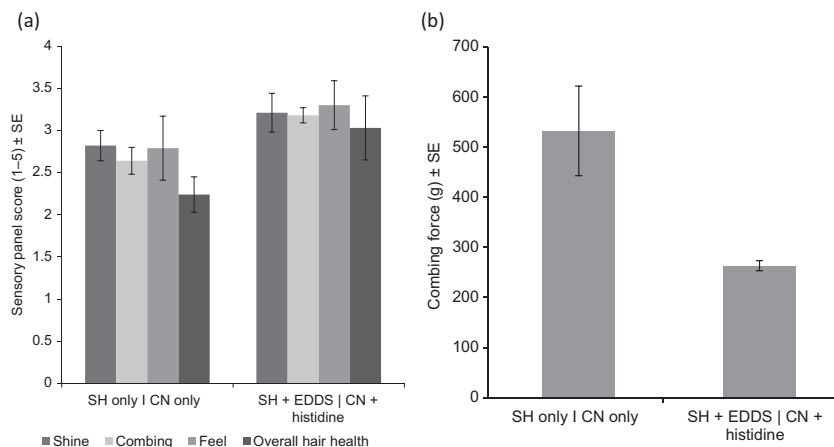


Figure 11 (a) Sensory evaluation of tresses following wear protocol; (b) combing force measurement of tresses following wear protocol.

tap water taken up by hair. It has been shown that one specific protein damaged by copper is S100A3, a calcium-binding protein located preferentially in the endo-cuticle. STEM-XEDS data confirmed that copper ions are concentrated in the sulphur-poor endo-cuticle and thus likely located close to the site of radical degradation for S100A3. Lipid degradation via copper is also suggested from lipid peroxide data leading to a proposal of two mechanisms for copper-induced radical formation via UV initiation that involves both hair proteins and lipids. A four-month wear study demonstrated that this structural degradation in combination with

physical damage from combing/washing, etc. will over time lead to loss of shine, poor combing and poor hair health. The use of chelants such as EDDS and histidine in shampoos and conditioners, respectively, will reduce copper levels in hair and ultimately lead to improved hair health.

Acknowledgements

The authors would like to thank The Procter & Gamble Company for support of this work.

References

- Pande, C.M. and Jachowicz, J. Hair photo-damage – measurement and prevention. *J. Cosmet. Sci.* **44**, 109–122 (1993).
- Nogueira, A.C.S., Dicelio, L.E. and Joekes, I. About photodamage of human hair. *Photochem. Photobiol. Sci.* **5**, 165–169 (2006).
- Santos Nogueira, A.C., Nakano, A.K. and Joekes, I. Impairment of hair mechanical properties by sun exposure and bleaching treatments. *J. Cosmet. Sci.* **55**, 533–537 (2004).
- Hoting, E. and Zimmerman, M. Photochemical alteration in human hair. Part III: investigation of internal lipids. *J. Cosmet. Chem.* **47**, 201–211 (1996).
- Korytowski, W., Pilas, B., Sarna, T. and Kalanaraman, B. Photo induced generation of hydrogen peroxide and hydroxyl radicals in melanins. *Photochem. Photobiol.* **45**, 185–190 (1987).
- Santos Nogueira, A.C. and Joekes, I. Hair color changes and protein damage caused by ultra violet radiation. *J. Photochem. Photobiol. B Biol.* **74**, 109–117 (2004).
- Godfrey, S., Staitte, W., Bowtell, P. and Marsh, J. Metals in female scalp hair globally and its impact on perceived hair health. *Int. J. Cosmet. Sci.* **35**, 264–271 (2013).
- Marsh, J.M., Iveson, R., Flagler, M.J. et al. Role of copper in photochemical damage to hair. *Int. J. Cosmet. Sci.* **36**, 32–38 (2014).
- Kizawa, K., Inoue, T., Yamaguchi, M., Kleinert, P., Troxler, H., Heizmann, C.W. and Iwamoto, Y. Dissimilar effect of perming and bleaching treatments on cuticles: advanced hair damage model based on elution and oxidation of S100A3 protein. *J. Cosmet. Sci.* **56**, 219–226 (2005).
- Kizawa, K., Uchiwa, H. and Murakami, U. Highly-expressed S100A3, a calcium-binding protein in human hair cuticle. *Biochim. Biophys. Acta* **1312**, 94–98 (1996).
- Gilchrist, J.B., Basey-Fisher, T., Chang, S.C.E., Scheltens, F., McComb, D.W. and Heutz, S. Uncovering buried structure and interfaces in molecular photovoltaics. *Adv. Funct. Mater.* **24**, 6473–6483 (2014).
- Allen, L.J., D'Alfonso, A.J., Freitag, B. and Klenov, D.O. Chemical mapping at atomic resolution using energy-dispersive x-ray spectroscopy. *MRS Bull.* **37**, 47–52 (2012).
- Fernández, E., Barba, C., Alonso, C., Martí, M., Parra, J.L. and Coderch, L. Photodamage determination of human hair. *J. Photochem. Photobiol. B: Biol.* **106**, 101–106 (2012).
- Takizawa, T., Takizawa, T., Arai, S., Kizawa, K., Ichiwa, H., Sasaki, I. and Inoue, T. Ultrastructural localization of S100A3, a cysteine-rich calcium binding protein, in human scalp hair revealed by rapid-freezing immunocytochemistry. *J. Histochem. Cytochem.* **47**, 525–532 (1999).
- Inoue, T., Sasaki, I., Yamaguchi, M. and Kizawa, K. Elution of S100A3 from hair fiber: new model for hair damage emphasizing the loss of S100A3 from cuticle. *J. Cosmet. Sci.* **51**, 15–25 (2000).
- Robbins, C.R. The cell membrane complex: three related but different cellular cohesion components of mammalian hair fibers. *J. Cosmet. Sci.* **60**, 437–465 (2009).
- Pan, Y., Zhang, L. and Liu, L. XAFS study of coordination structure of Cu(L-His)₂ in solution. *Chin. J. Chem. Phys.* **24**, 451–456 (2011).