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Mapping Protein Cross-Links in Human Hair via Mass Spectrometry

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Synopsis

Networks of protein–protein cross-links underpin all the key physicomechanical properties of mammalian fibers and also strongly influence how the fiber responds to treatments and environmental insults. During the last decade, significant improvement has been made in detecting and understanding cross-links, though, to date, mapping where these protein cross-links are within and between proteins has proved to be very challenging. This work reports a proof-of-concept study where mass spectrometry–based proteomics strategies were used to unravel the details of cross-link location between trichocyte keratin proteins in the hair shaft. This work focuses on two cross-links known to exist in hair proteins and that are used as a proxy for the degree of damage in hair proteins: lanthionine and lysinoalanine. Our results demonstrate that mass spectrometric evidence of the existence of both lanthionine and lysinoalanine cross-linked peptides within hair fibers can be found. The approach used also provided the first insight of the exact location of these cross-links within specific keratins. Analysis with respect to the current models of trichocyte intermediate filament organization indicates detected cross-links occur within antiparallel keratin heterodimers. The low abundance of the cross-linked peptides makes this type of evaluation approach difficult, but the results represent a new approach to untangle which cross-links are most essential to defining hair performance.

INTRODUCTION

Human hair is a complex tissue with fibers consisting of three morphological compartments composed of cornified dead cells: the cuticle, the cortex, and the medulla. The medulla is a loosely packed region in the center of the hair shaft, surrounded by the cortex, which represents the major structure in hair. To protect the cortex against the outside environment, a layer of overlapping cells called the cuticle surrounds the cortex (1). The hair cortex is primarily composed of proteins (90–95%), with lipids, water, and trace elements as the other chemical constituents present. The cortical proteome is dominated by two classes of proteins: first, intermediate filament–forming trichocyte (or alpha) keratins, and second, keratin-associated proteins that surround the keratin filaments. The keratins associate

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together to form coiled-coil dimers, intermediate filaments, and macrofibrils (2). To achieve a rigid hair structure, they form a strong compact network by both intra- and intermolecular interactions such as disulfide bonds, hydrogen bonds, electrostatic salt bonds, and amide bonds (3). Of major interest in hair science are the protein cross-links, such as disulfide bonds, which lead to the formation of covalent linkages between intermolecular amino acid residues and hence determine which regions within peptides and proteins are involved in the formation of protein networks. In addition to these native protein cross-links in hair, artificial cross-links, including lanthionine and lysinoalanine, can also be induced, mostly on insult such as heat, UV, or the use of alkaline products (4).

Networks of protein–protein cross-links underpin all the key physicomechanical properties of mammalian fibers and strongly influence how the fiber responds to treatments and environmental insults. Understanding and controlling these networks is therefore critical to enhance our understanding of fiber science generally and develop new fiber treatments that enhance properties. However, due to the large degree of cross-linking present in mammalian fibers, analysis of these covalent bonds has been very difficult. Although some technologies have successfully led to the detection of cross-links (3), most techniques involve total protein destruction, making mapping where these cross-links are within and between proteins extremely difficult.

In this proof-of-concept study, mass spectrometry–based proteomics strategies are used to unravel the details of cross-link location between trichocyte keratin proteins in the hair shaft. This work focuses on two cross-links known to exist in hair proteins and that are used as a proxy for the degree of damage in hair proteins: lanthionine and lysinoalanine. Both cross-links can be the result of degraded disulfide bonds to form dehydroalanine, or alternatively, of the elimination of water or hydrogen sulfide from serine and cysteine, respectively, to form dehydroalanine. This dehydroalanine intermediate will react with either lysine to form lysinoalanine or with cysteine to from lanthionine. Although mass spectrometry has been used before to elucidate protein cross-links in a food or medical context (5,6), this work, to our knowledge, is the first to decipher cross-links in the complex hair matrix with the aim of finding mass spectrometric evidence of the existence of cross-links within hair fibers and mapping their exact location within the protein *in situ*.

METHODS

SAMPLE PREPARATION

Blended Caucasian virgin hair tresses were obtained from International Hair Importers & Products, Inc. (Glendale, NY, United States). Full-length fibers were cut into snippets of approximately 5 mm in length.

For amino acid analysis, two types of samples were prepared: first, 10 mg of hair snippets were weighted, and second, hair protein extracts were prepared by using 100 mg of hair snippets, which were dissolved in 5 mL of an extraction buffer (7 M urea, 2 M thiourea, 0.05 M Tris, and 50 mM dithiothreitol (DTT), pH 7.5) at room temperature for 18 h using vigorous shaking. For proteomics analysis, 10 mg of hair snippets were dissolved in 1 mL of an extraction buffer (7 M urea, 2 M thiourea, 0.05 M Tris, and 50 mM DTT, pH 7.5) at room temperature for 18 h using vigorous shaking.

PROTEIN DIGESTION

After the protein extraction, an acetone precipitation was carried out to remove the extraction buffer components by adding 5 mL of ice-cold acetone to 1 mL of the sample. The samples were stored at –20°C for 48 h before they were centrifuged at 10,000 *g* and 4°C for 10 min. The protein pellet was washed twice with ice-cold acetone, air-dried, and resuspended in 200 μL of 10 mM ammonium bicarbonate. The protein concentration per sample was determined by Direct Detect (Millipore, MA, United States) according to manufacturer's instructions. Next, 100 μg of each sample was aliquoted, and proteins were digested by sequencing grade trypsin (Promega) at a 1:50 ratio (trypsin:protein) for 18 h at 37°C at 300 rpm in a thermomixer. Next, the tryptic digest peptide mixtures were stored at –20°C until further analysis.

AMINO ACID ANALYSIS

The hair snippets and the tryptic digests were hydrolyzed with 6 N HCl at 110°C for 24 h. Amino acid analysis was performed using a Dionex HPLC system (Dionex UltiMate 3000 with Fluorescence Detector, Thermo Scientific, San Jose, CA, United States). Free amino acid residues and standards (containing lanthionine and lysinoalanine at a predetermined concentration) were derivatized with an AccQ-Tag reagent (Waters Corporation, Milford, MA, United States) and separated using a Thermo Accucore XL C18 column (4.6 mm i.d. × 250 mm, 4 μm particles) (Thermo Scientific, San Jose, CA, United States) at 37°C using a flow rate of 1.0 mL/min. Eluent A was AccQ-Tag solvent A, and eluent B was 100% acetonitrile. An excitation wavelength of 250 nm and emission wavelength of 395 nm were used for the quantitative analysis using a fluorescence detector (Dionex Ultimate FLD 3000, Thermo Scientific, San Jose, CA, United States).

LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

To detect the cross-links via mass spectrometry, the peptide mixtures were separated on an Ultimate 3000 RSLCnano system (Thermo Scientific, San Jose, CA, United States) using a C18 PepMap100 nano-Trap column (200 μm i.d. × 2 cm) (Thermo Scientific, San Jose, CA, United States) connected to a ProntoSIL C18AQ analytical column (100 μm i.d. × 15 cm, 3 μm particle size, 200 A pore size) (nanoLCMS Solutions, Gold River, CA, United States). For each sample 1 μL (equal to 1 μg) was loaded on the trap column at 3 μL/min. The trap column was then switched in line with the analytical column. Reverse phase liquid chromatography (LC) separation was performed by a linear gradient of mobile phase B (0.1% formic acid in 100% acetonitrile) from 2% to 45% in 60 min, followed by a steep increase to 98% mobile phase B in 6 min, held at 95% mobile phase B for 2 min, returned to 2% mobile phase B over 5 min, and then re-equilibrated at 2% mobile phase B for 15 min resulting in a total run of 88 min used at a flow rate of 1,000 nL/min. The LC was coupled online to mass spectrometry via a Bruker CaptiveSpray ion source (Bruker Daltonics, Bremen, Germany) equipped with a nanobooster device and operated at 1,400 V.

Data were acquired with a Q-TOF Impact II (Bruker Daltonics, Bremen, Germany) mass spectrometer in a dynamic data-dependent auto-MS/MS mode where a full scan spectrum (150–2,200 *m/z*, 2 Hz) was followed by a dynamic inclusion of collision-induced dissociation

tandem mass spectra (2–32 Hz, depending on intensity) for 3 s. A preference of selection of multiple-charged precursors (2+ to 5+) was made, and an isolation width of 0.03 Th was set. Dynamic exclusion duration was set to 20 s.

PROTEIN IDENTIFICATION

Potential protein–protein cross-links were searched with Kojak v1.6 (7). The following parameters were used: a precursor mass tolerance of 10 ppm was allowed with fragment bin offset = 0, fragment bin size 0.03. A custom in-house database was created containing all human keratin protein sequences, keratin-associated proteins, and their reverse complements as decoy proteins. Trypsin was specified as a digestive enzyme, and up to three missed cleavages were allowed. A minimum peptide mass of 245 Da was set. Carbamidomethyl (C), oxidation (M), and deamidation (NQ) were chosen as variable modifications, and a maximum of three modifications per peptide was allowed. A lanthionine bond (Cys–Cys or Ser–Cys) or lysinoalanine bond (Lys–Cys) was specified as a cross-link. For each spectrum, the top 20 scoring peptides were used for cross-link combination. To minimize false positives, peptide spectrum matches exported from Kojak were further validated by Percolator V2.08 (8). The threshold for peptide spectrum matches after validation was set at q value smaller than 0.01.

RESULTS

CONFIRMATION OF CROSS-LINK PRESENCE IN HAIR FIBERS AND THEIR PROTEIN EXTRACTS

Amino acid analysis was used to validate the presence of lysinoalanine or lanthionine cross-links in samples that were used for proteomics cross-link mapping. The amount of lanthionine and lysinoalanine present both in hair fiber snippets (one biological replicate run in duplicate) and hair snippet protein extracts (three separate protein extracts run in duplicate) is shown in Figure 1. Cross-links were detected in both the fiber and in the protein extract from the fiber, though some variability in the samples was noticed.

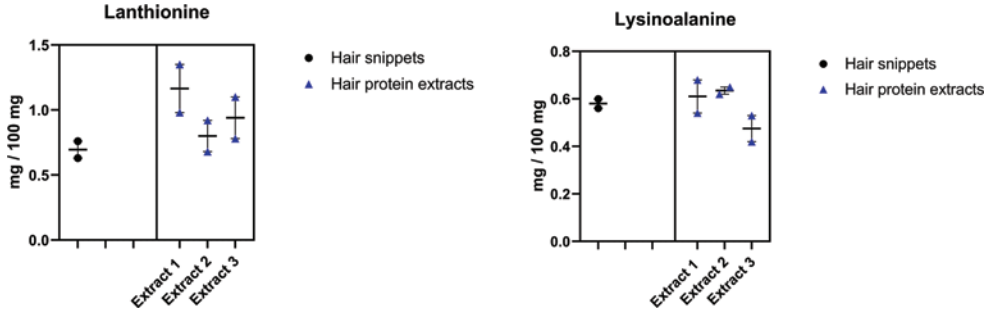


Figure 1. The quantities of lanthionine and lysinoalanine cross-links (mg/100 mg) present in hair snippets (black) and triplicate hair extracts (blue) as determined by amino acid analysis represented with 95% confidence intervals.

LOCATION OF LANTHIONINE CROSS-LINKS WITHIN HAIR PROTEIN EXTRACTS VIA MASS SPECTROMETRY

As cross-linked peptides are very challenging to characterize by mass spectrometry, a data acquisition method that allows for better fragmentation of these low-abundant cross-linked peptide precursors was developed. By allowing a more dynamic sampling of precursors in the mass spectrometer, a higher chance of detecting low-abundant, multiple-charged precursors was created.

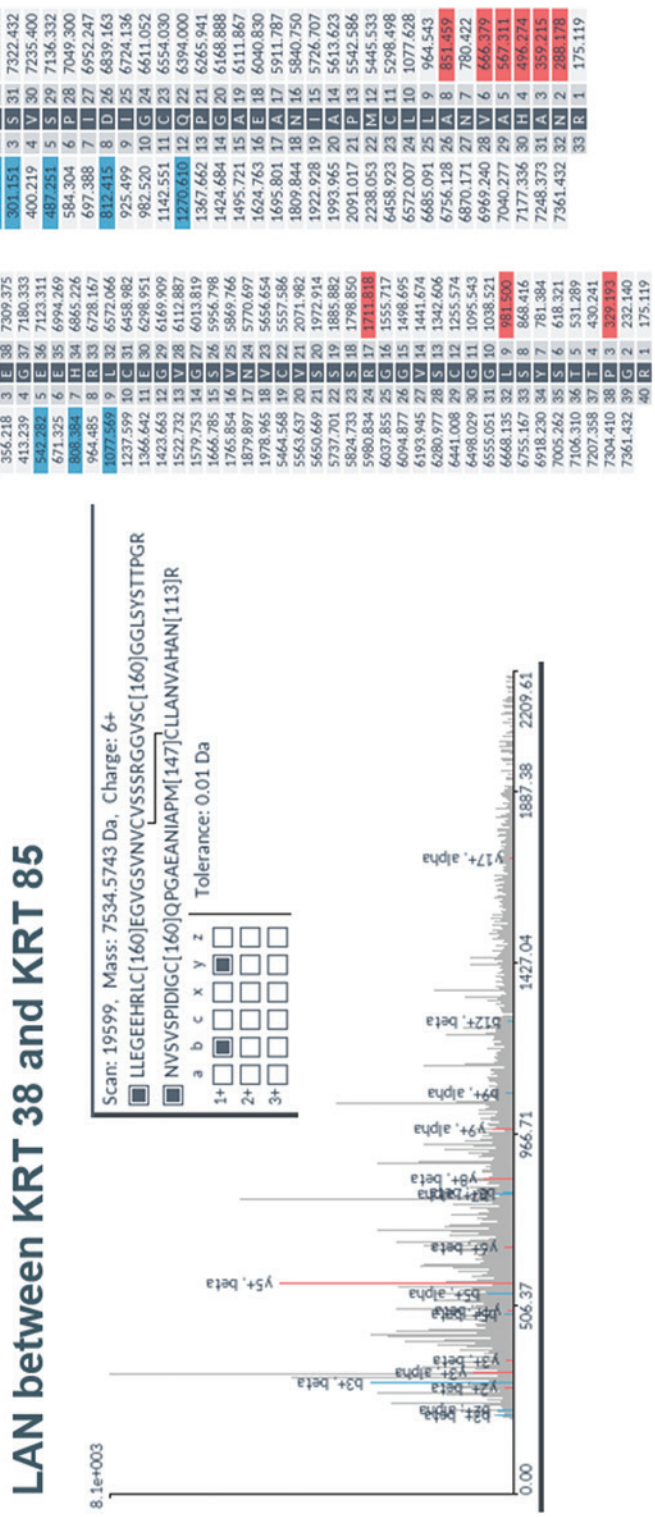
To maximize the chance of detecting lanthionine and lysinoalanine cross-links between keratin-related proteins and to minimize the amount of false positive hits within this process, we designed a custom database that consisted of all human keratins and keratin-associated proteins, as it is believed that cross-links formed within these protein families are largely responsible for the hair fiber rigid structure. Using this FASTA file (2019.02), we analyzed for cross-links between 298 proteins (including decoy proteins), resulting in 22,204 *in silico* tryptic peptide combination possibilities, of which 8,759 peptides are linkable.

Table I lists lanthionine cross-links that could be detected in the hair digests. The table lists the two potential peptide sequences that are linked by a lanthionine cross-link as well as their corresponding first hit proteins. It also provides the spectrum number so that further manual validation of the fragmentation spectrum is possible. The spectrum-match validation settings were chosen as very stringent, and only q values smaller than 0.05 were considered confident. Because the algorithm only reports q values to three decimal places, the q values that made it through the filter are reported as 0.

A further look at the fragmentation spectrum of the detected cross-link is depicted in Figure 2. A few observations can be made. First, as lanthionine derives from dehydroalanine intermediates (6), the bond can exist between cysteine–cysteine or serine–cysteine in the cross-linked peptides. All the cross-linked peptides detected consisted of a cysteine–cysteine bond. Second, none of the annotated fragment ions are ions that are directly linked to the Cys–Cys bond, though several other ions are detected. Third, the detected fragment ions are still all very low abundance, yet through our dynamic acquisition method, just detectable. Fourth, the detected b-ions and y-ions originating from the two peptides, the dominant alpha peptide, as well as the beta peptide are indicated.

Table I
List of Lanthionine Cross-Links Found in Human Virgin Hair Shaft Digest

Scan number	q value	Peptide	Peptide	Protein 1	Protein 2
19,599	0	.LLEGEEHRLC[57.02]EGVGSVN VCVSSSRGGVSC[57.02]GGLSYSTTPGR	NVSVSPIDIGC[57.02]QPGAEAN IAPM[15.99]CLLANVAHAN[0.98]R	HUMAN_K85	HUMAN_K38
6,471	0	.QVVSSSEQLQ[0.98]SCQAEIIELR	ISSGCGVTRNFSSCSAVAPK	HUMAN_K34	HUMAN_K85
4,490	0	.SQQQEPLVCASYQSYFK	CEIGNVK	HUMAN_K33A	HUMAN_K74
4,479	0	.SQQQEPLVCASYQSYFK	CEIGNVK	HUMAN_K33A	HUMAN_K74
3,540	0	.SQQQEPLLCPSYQSYFK	MSCRSYR	HUMAN_K34	HUMAN_K84



b+	#		#	y+
114.091	1	L	40	
227.175	2	L	39	7422.459
356.218	3	E	38	7309.375
413.239	4	G	37	7180.333
542.282	5	E	36	7123.311
671.325	6	E	35	6994.269
808.384	7	H	34	6865.226
964.485	8	R	33	6728.167
1077.569	9	L	32	6572.066
1237.599	10	C	31	6458.982
1366.642	11	E	30	6298.951
1423.663	12	G	29	6169.909
1522.732	13	V	28	6112.887
1579.753	14	G	27	6013.819
1666.785	15	S	26	5956.798
1765.854	16	V	25	5869.766
1879.897	17	N	24	5770.697
1978.965	18	V	23	5656.654
2064.568	19	C	22	5557.586
2163.637	20	V	21	2071.982
2250.669	21	S	20	1972.914
2337.701	22	S	19	1885.882
2424.733	23	S	18	1798.850
2580.834	24	R	17	1711.818
2637.855	25	G	16	1555.717
2694.877	26	G	15	1498.695
2793.945	27	V	14	1441.674
2880.977	28	S	13	1342.606
2941.008	29	C	12	1255.574
2998.029	30	G	11	1095.543
3055.051	31	G	10	1038.521
3168.135	32	L	9	981.500
3355.167	33	S	8	868.416
3418.230	34	Y	7	781.384
3505.262	35	S	6	618.321
3606.310	36	T	5	531.289
3707.358	37	T	4	430.241
3804.410	38	P	3	329.193
3861.432	39	G	2	232.140
	40	R	1	175.119

b+	#		#	y+
115.050	1	N	33	
214.119	2	V	32	7421.501
301.151	3	S	31	7322.432
400.219	4	V	30	7235.400
487.251	5	S	29	7136.332
584.304	6	P	28	7049.300
697.388	7	I	27	6952.247
812.415	8	D	26	6839.163
925.499	9	I	25	6724.136
982.520	10	G	24	6611.052
1142.551	11	C	23	6554.030
1270.610	12	Q	22	6394.000
1367.662	13	P	21	6265.941
1424.684	14	G	20	6168.888
1495.721	15	A	19	6111.867
1624.763	16	E	18	6040.830
1695.801	17	A	17	5911.787
1809.844	18	N	16	5840.750
1922.928	19	I	15	5726.707
1993.965	20	A	14	5613.623
2091.017	21	P	13	5542.586
2238.053	22	M	12	5445.533
6458.923	23	C	11	5298.498
6572.007	24	L	10	1077.628
6685.091	25	L	9	964.543
6756.128	26	A	8	851.459
6870.171	27	N	7	780.422
6969.240	28	V	6	666.379
7040.277	29	A	5	567.311
7177.336	30	H	4	496.274
7248.373	31	A	3	359.215
7361.432	32	N	2	288.178
	33	R	1	175.119

Figure 2. Mass spectrometric evidence of the existence of lanthionine inter–cross-links between two peptides from keratin 38 and keratin 85. The data visualize scan number 19,599 of Table I.

A further look at the localization of the two peptides (red) within the full sequences of the respective proteins is visualized in Figure 3. Interestingly, the peptides of interest are mapped at opposite ends of the total sequence.

An analogous analysis was performed for the other potential cross-links detected. The lanthionine link between keratin 34 and keratin 85 is visualized in Figure 4. Similar to the first example, no direct proof of the cross-link via a fragment ion could be detected, though proof of existence of both peptide sequences (the alpha and beta sequence) within the fragment spectrum can be found (Figure 4). Although the cross-linked peptides between keratin 33A and keratin 74 were found in two spectra, most ion intensities were again very low; this might indicate that the bulk of the ion intensities are still too low to be confidently detectable by our method.

DETECTION OF LYSINOALANINE CROSS-LINKS WITHIN HAIR FIBER PROTEIN EXTRACTS VIA MASS SPECTROMETRY

A similar approach was also taken to explore whether there is mass spectrometric evidence of lysinoalanine bonds between keratins and/or keratin-associated proteins present in the tryptic digest of the extracted hair fiber proteins. Table II lists all lysinoalanine cross-links found with q values smaller than 0.05.

From this list, two cross-links of interest were further investigated on their potential. At first, mass spectrometric evidence was sought for the link between keratin 33B and keratin 80. Figure 5 displays the fragment spectrum with the detected b-ions and y-ions in blue and red, respectively. Figure 6 represents the location of the two peptides (red) within the full sequences of the respective proteins. Similar to the lanthionine data, proof of presence of both peptides was found as ions from both the alpha peptide and beta peptide were detected.

A closer look at the potential lysinoalanine cross-link between keratin 34 and keratin 82, alternatively, shows that most ions are found with a very low signal-to-noise threshold (Figure 7), indicating that the interpretation of these data should be approached with extra caution.

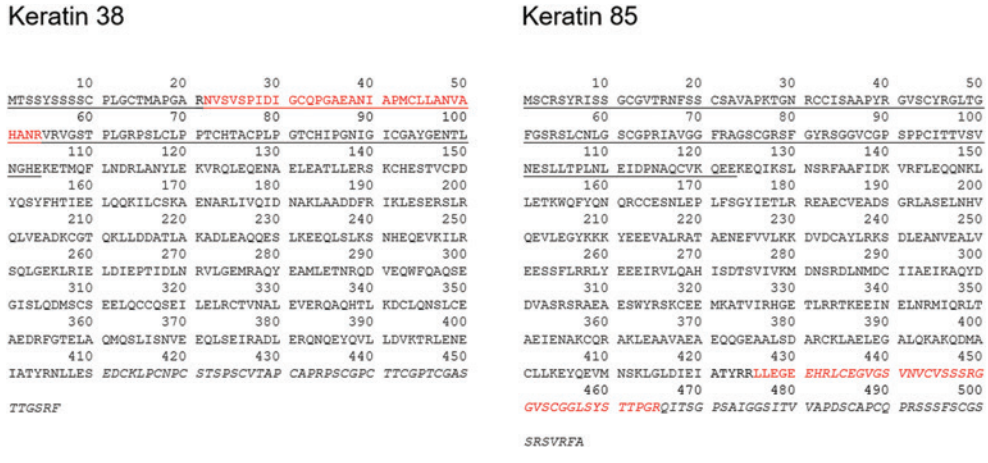


Figure 3. Protein sequences (human) of keratin 38 and keratin 85, with the peptide sequence involved in the cross-link indicated in red, the head domain underlined, and the tail domain in italics.

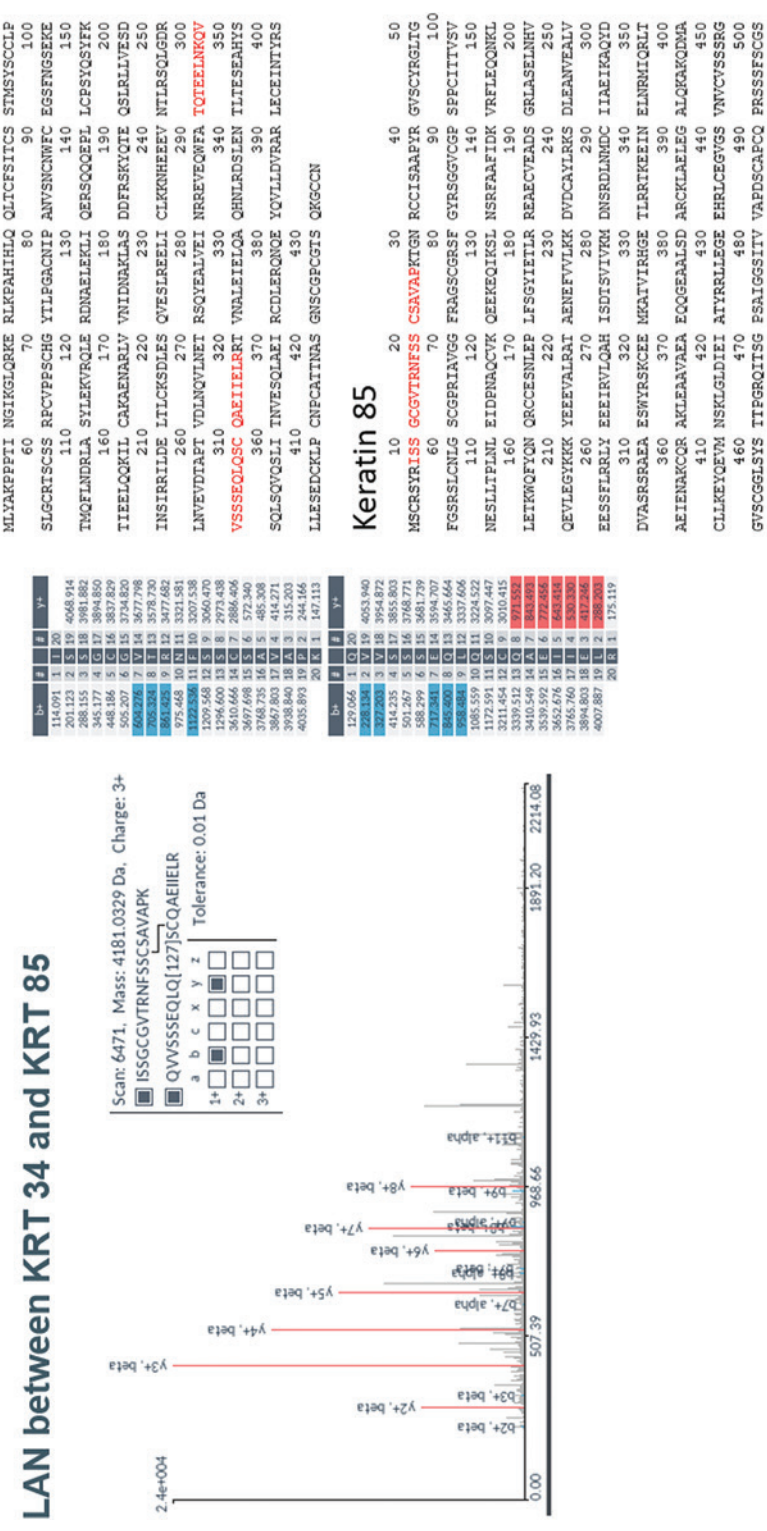


Figure 4. Another example of mass spectrometric characterization of an intermolecule lanthionine cross-link found between keratin 34 and keratin 85 within a tryptic digest of hair shaft-extracted proteins.

Table II
List of Lysinoalanine Cross-Links Found in Human Virgin Hair Shaft Digest With *q* Smaller Than 0.05

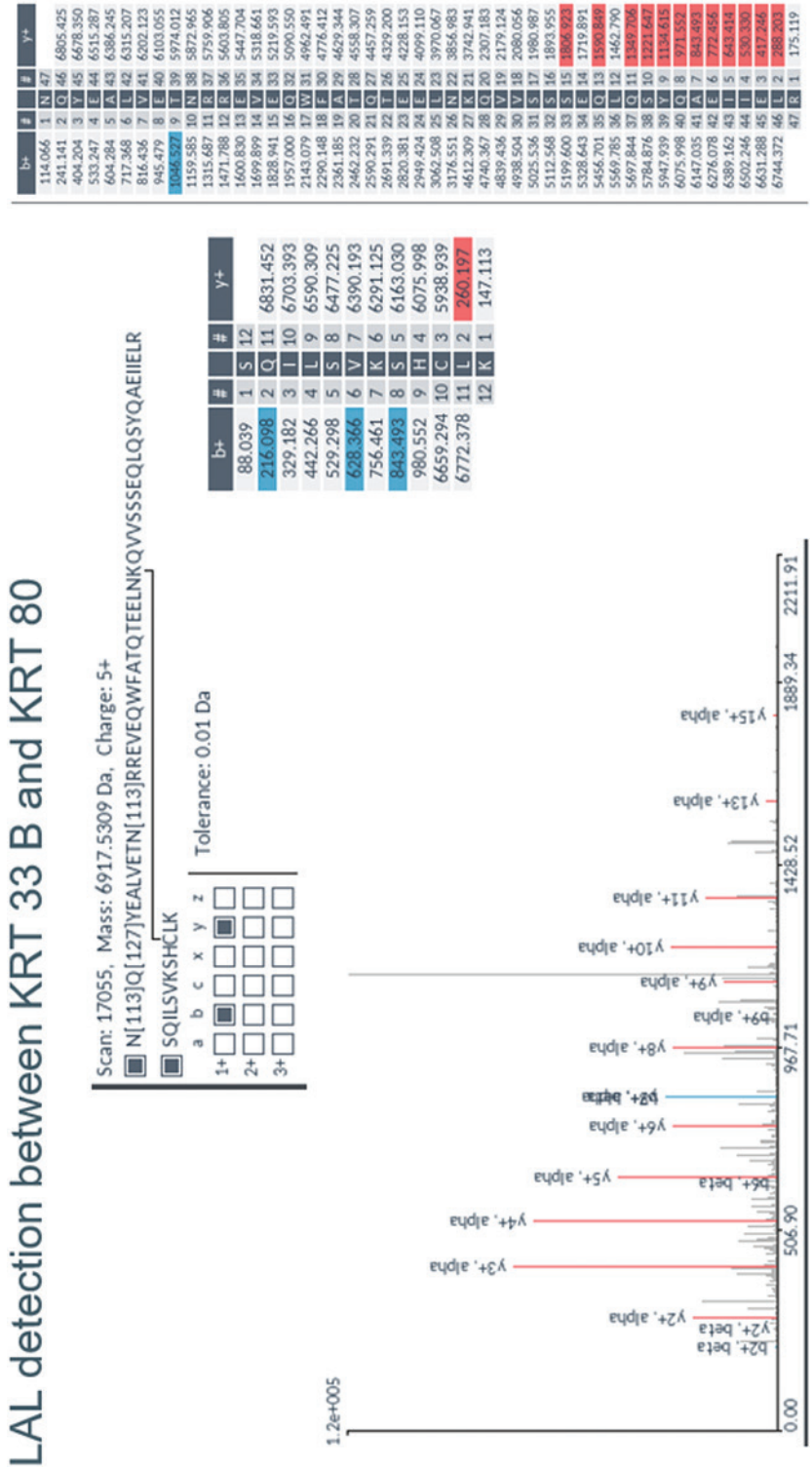
Scan number	*q* value	Peptide 1	Peptide 2	Protein 1	Protein 2
8040	0	.ADLETNAEALVQEIDFL KSLYEEEICLLQSQ[0.98] ISETSVIVKMDNSR	TMQDSVEDF KTKYEEEEINK	HUMAN_K4	HUMAN_K82
19330	0	.GPC[57.02] RPGGGRGLRALG CLGSRSLCN[0.98] VGFGRPR	KN[0.98]HEEEVN [0.98]TLR	HUMAN_K82	HUMAN_K34
18602	0	.KDVDCAYLRKSDLEANVE ALVEESSFLR	KVLEEVDQR	HUMAN_K85	HUMAN_K25
17055	0.030	.N[0.98]Q[0.98]YEALVETN [0.98]RREVEQWFA TQTEELNKQVVSSSEQ LQSYQAEIIELR	SQILSVKSHCLK	HUMAN_ K33B	HUMAN_K80
17824	0	.SQYEALVETN RREVEQWFATQ[0.98] TEELNKQVVS SSEQLQSYQAEIIELR	SRQFTCKSGAAAK	HUMAN_ K33A	HUMAN_K71

DISCUSSION

Intermediate filaments are a key repeating structural unit of the human hair shaft, and their stability depends on cross-links both within filaments and between filaments via a network that extends through the interfilament matrix. The premise for this study was that the protein–protein cross-link network underpins health- and consumer-relevant physicomechanical properties of hair and that some cross-links are more important than others in their contribution to these properties. Location of individual cross-links is valuable to better understand the role of specific cross-links on properties and to investigate macromolecular relationships within and between keratin intermediate filaments.

In the last few decades, significant progress has been made in understanding the exact structure of the intermediate filaments through insights gained via amino acid analysis, computer modeling, x-ray fiber diffraction and protein crystallography, electron microscopy, and chemical cross-linking methods (2,9). These methods have helped us understand the structural detail of the hierarchical structure of keratins into heterodimers, tetramers, and 32-chain intermediate filaments. Currently, however, widespread pinpointing of the exact location of cross-links within and between the intermediate filament proteins is still very challenging. In this proof-of-concept study, we have, for the first time, applied mass spectrometric techniques to detect native cross-links present within the human hair cortex and to map the exact location of these cross-links within the proteins.

The use of mass spectrometry in protein cross-links has challenges, including, but not limited to, low abundance of cross-linked peptides, fragmentation of the cross-link peptides by the mass spectrometer, and the lack of automated data analysis for cross-linked peptides (5,6). Therefore, most of the work published on native protein cross-links to date involves a noncomplex mixture of proteins. Extending this technology to a complex hair shaft proteome comes with challenges in data complexity. Although disulfide bonds are the best characterized cross-links between keratin proteins in hair, their analysis in complex samples is complicated because typical proteomics approaches break disulfide bonds to



b+	#		#	y+
88.039	1	S	12	
216.098	2	Q	11	6831.452
329.182	3	I	10	6703.393
442.266	4	L	9	6590.309
529.298	5	S	8	6477.225
628.366	6	V	7	6390.193
756.461	7	K	6	6291.125
843.493	8	S	5	6163.030
980.552	9	H	4	6075.998
6659.294	10	C	3	5938.939
6772.378	11	L	2	260.197
	12	K	1	147.113

b+	#		#	y+
114.066	1	N	47	
241.141	2	Q	46	6805.425
404.204	3	Y	45	6678.350
533.247	4	E	44	6515.287
604.284	5	A	43	6386.245
717.368	6	L	42	6315.207
816.436	7	V	41	6202.123
945.479	8	E	40	6103.055
1086.5927	9	T	39	5974.012
1159.585	10	N	38	5872.965
1315.687	11	R	37	5759.906
1471.788	12	R	36	5603.805
1600.830	13	E	35	5447.704
1699.899	14	V	34	5318.661
1828.941	15	E	33	5219.593
1957.000	16	Q	32	5090.550
2143.079	17	W	31	4962.491
2290.148	18	F	30	4776.412
2361.185	19	A	29	4629.344
2462.232	20	T	28	4558.307
2590.291	21	Q	27	4457.259
2691.339	22	T	26	4329.200
2820.381	23	E	25	4228.153
2949.424	24	E	24	4099.110
3062.508	25	L	23	3970.067
3176.551	26	N	22	3856.983
4612.309	27	K	21	3742.941
4740.367	28	Q	20	2307.183
4839.436	29	V	19	2179.124
4938.504	30	V	18	2080.056
5025.536	31	S	17	1980.987
5112.568	32	S	16	1893.955
5199.600	33	S	15	1806.923
5328.643	34	E	14	1719.891
5456.701	35	Q	13	1590.849
5569.785	36	L	12	1462.790
5697.844	37	Q	11	1349.706
5784.876	38	S	10	1221.647
5947.939	39	Y	9	1134.615
6075.998	40	Q	8	971.552
6147.035	41	A	7	843.493
6276.078	42	E	6	772.456
6389.162	43	I	5	643.414
6502.246	44	I	4	530.330
6631.288	45	E	3	417.246
6744.372	46	L	2	288.203
	47	R	1	175.119

Figure 5. Mass spectrometric characterization of intermolecule lysinoalanine cross-links found between keratin 33B and keratin 80 within a tryptic digest of hair shaft-extracted proteins.

Figure 6. Protein sequences (human) of keratin 33B and keratin 80, with the peptide sequence involved in the cross-link indicated in red.

unfold proteins to enable enzymatic digestion and mass spectrometric analysis (10). This proof-of-concept study focused on lanthionine and lysinoalanine cross-links induced by environmental wear and tear. To ensure that the target cross-links were present in our samples, amino acid analysis against lanthionine and lysinoalanine standards measured the absolute quantity of cross-links present in hairs and protein extracts. Although amino acid analysis did not indicate the molecular location of target cross-links, it was essential to creating a robust mass spectrometry and analysis method.

In this work, mass spectrometric data acquisition and further analysis of the hair protein extracts did offer additional insights about the approach we developed for mapping cross-links:

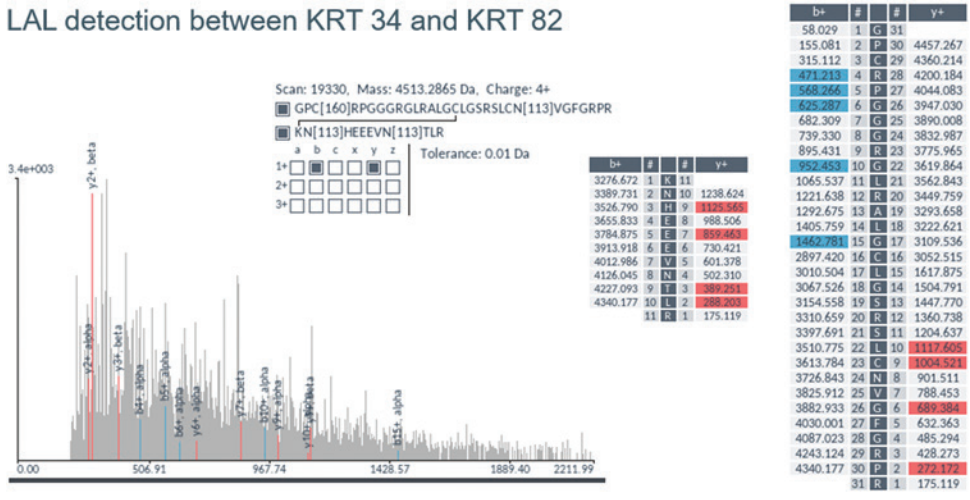


Figure 7. Mass spectrometric characterization of intermolecule lysinoalanine cross-links found between keratin 34 and keratin 82 within a tryptic digest of hair shaft-extracted proteins. The signal-to-noise level of this cross-link is very low, and interpretation of the data should be done with caution as hardly any difference between the target ions and background ions is seen.

1. First, though many cross-linked peptide ions can still not be detected due to the complexity of the sample, this study provides a first proof-of-principle that our approach, which targeted lanthionine and lysinoalanine cross-links, proved successful for mapping locations within human hair proteins. In each case, fragment ions that match both linked peptides were detected.
2. Second, the high homology in protein sequences in the keratin family complicates the identification of cross-linked proteins. We chose to report only the "first hit" protein (i.e., the protein with the highest score) during database matching. However, for some peptides it is possible that the peptide sequence of interest is not unique to just one protein.
3. Third, false positive cross-link identifications during data analysis require careful consideration. While our initial results mapped cross-links to both keratins and keratin-associated proteins, after applying stringent data analysis settings to minimize false positive identifications, in this work only linkages between keratins were confidently identified.

POSITION OF THE CROSS-LINK WITHIN THE FIBER

Next these preliminary mass spectrometric data were compared to the current models of trichocyte intermediate filament organization. Intermediate filament–forming keratin proteins can be classified into two major types: acidic type I keratins and neutral-basic type II keratins. Within the hair cortex, a type I and type II keratin form a coiled-coil heterodimer. To keep this three-dimensional coiled structure of the intermediate filament stable, protein–protein cross-links are crucial. Tetramers are formed when two of these heterodimers are clustered in an antiparallel fashion (9,11,12). Protofilaments cluster different tetramers, which end up in the intermediate filament structure (Figure 8). Three

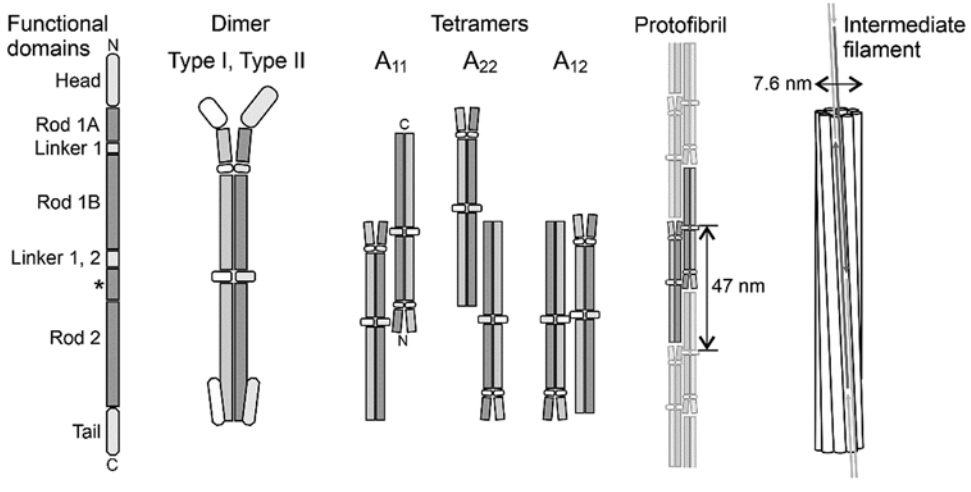


Figure 8. Schematic representation of the trichocyte keratin intermediate filament molecule. Each molecule is a heterodimer containing a type I and type II chain keratin. Tetramers are formed when two of these heterodimers are clustered in an antiparallel fashion. In these tetramers, three modes are common to all classes of intermediate filaments and involve (respectively) the approximate axial alignment of antiparallel 1B segments, antiparallel 2 segments, and antiparallel rod domains. Protofibrils cluster different tetramers, which end up in the intermediate filament structure.

different modes of molecular assembly of intermediate filaments are described. These three modes are common to all classes of intermediate filaments and involve (respectively) the approximate axial alignment of antiparallel 1B segments (model A_{11}), antiparallel 2 segments (model A_{22}), and antiparallel rod domains (model A_{12}) (Figure 8). Previous work from the Parry et al. research groups (11,13–16) do show that newly assembled protofilament molecules associate in A_{11} or A_{22} alignments within the follicle before a profound structural rearrangement occurs when the environment changes from a reducing to an oxidizing one.

We compared these filament models with our experimental data to get a better idea of what these positions of the cross-linked peptides within the protein sequence mean, and to see if our data aligned with the models of trichocyte intermediate filament organization. Important to note, though, is that this work is not intending to make any assumptions on the structural intermediate filament biology aspect.

The lanthionine cross-link found between keratin 38 and keratin 85 matches the models in a number of ways: first, proteins keratin 38 and keratin 85 are, respectively, a type I and type II keratin and could thus form a heterodimer. They are both cortex proteins. Second, the position of the cross-linked peptides (Figure 3) demonstrates that the link is made between proteins in antiparallel positioned dimers forming a tetramer, with an interaction between a head domain of one protein and a tail domain of another. This could be an indication of the suggested model A_{12} in a tetramer but could also be the packaging of adjacent tetramers. Third, the A_{12} arrangement is probably the most prevalent in a mature hair fiber as it lends itself to optimal molecular packing and radial compaction in an oxidized environment (13).

Subsequently, we researched whether the other detected lanthionine cross-links could also be explained by these models. Similar to the previous example, the cross-link between keratin 33A and keratin 74, detected in two spectra (Table I), did show an A_{12} arrangement, with peptide 1 (AA 91–108) from keratin 33A located in linker 1, while peptide 2 from keratin 74 with amino acid position 370–378 in the sequence is part of coil 2. As these two proteins do not tend to be found in the same part of the fiber (i.e., K33a is in the cortex and medulla, while K74 is found in the inner root sheet), it is still a question of where this cross-link would be formed, as both peptides are unique and thus only identified in these respective proteins. The mass spectrometric evidence of the lanthionine cross-link between keratin 34 and keratin 85, a type I and type II keratin, does show a different positioning of the cross-linked peptides. Here, one peptide is found at the N-terminus (head region), while the other peptide is found in the linker 1 region (Figure 5). This lanthionine link would thus suggest that these two proteins are more comparable to the A_{11} model, though the position of the head sequence in the intermediate filament is still questionable.

Similarities between the proposed intermediate filament models are also found when looking at the trichocyte keratin lysinoalanine cross-links within the samples. Mass spectrometric mapping of a lysinoalanine cross-link between keratin 33B and keratin 80 displays an interesting link, as both proteins are present in the medulla. The medulla, however, is known to have a heterogenous structure, and hence, although the position of the peptides of interest shows that these are somewhat comparable to model A_{22}, where the proteins are still present in antiparallel heterodimers within the filament, these may or may not form tetramers in the medulla.

Though some mass spectrometric evidence for the presence of the other detected lysinoalanine cross-links is found, manual inspection of the data (e.g., the cross-link between keratin 34

and keratin 82 {Figure 7}) shows that the peptides are detected with fragments of very low intensity, and a distinction between the fragment ions and the background signals is hard to detect. Therefore, none of the other lysinoalanine data were taken forward for further comparison against the intermediate filament models.

In conclusion, our results demonstrate here, for the first time, the mass spectrometric characterization of native cross-links within hair fibers (lanthionine and lysinoalanine), combined with their mapping within the protein sequence, and their potential mapping within the intermediate filament. These results represent the first steps toward untangling the many unknowns related to cross-links in fibers. However, caution should still be taken, as our results clearly indicate that the low abundance of the cross-linked peptides still makes this type of evaluation approach difficult, even when optimized mass spectrometric data acquisition methods are applied. These preliminary data, however, do show evidence of previous reported heterodimer compositions and do showcase the potential of the technology.

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