

2018 IULTCS/Lear Corporation Young Leather Scientist Grant

What makes leather stronger? A mechanistic study on the effect of natural/artificial cross-links on tensile strength using small-angle neutron scattering (SANS)

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1. Introduction

Strength is a very important physical property of leather and determines its range of applications and value as a commercial product. To improve the strength of leather, aspects such as moisture level, weaving network, fibre coating, space filling and cross-linking chemistry need to be considered during the manufacturing.[1] As the major component of animal skins, the intermolecular structure of collagen has a strong relationship with the physical properties of leather. According to the hierarchical structure of collagen-based substrates, it is the precise staggering of triple helical collagen molecules that forms fibril, fibre, and finally, skin and leather.[2, 3] During the stretching of leather, fibre bundles will be pulled to a linear shape followed by the spread of tension to the lower level of hierarchical organisation - fibre, fibril and the individual tropocollagen molecules.[4] At the onset point of the stress-strain curve, the intermolecular force that retains the collagen molecular structure reaches the maximum load and then fails, and is observed as the breakage of the material in the macroscale.[4] During leather manufacturing, various types of tanning agents are used as cross-linkers to stabilize collagen. The formation of intermolecular cross-links can play an important role directly in contributing to the maximum stretching force that a single collagen fibril can withstand, as well as maintain its long-range ordered molecular packing structure.

Cross-links in tanned leather can be categorised by their origins: natural cross-links that exist inherently in the hides and skins, and artificial cross-links introduced during the tanning processes. Natural cross-links are a series of tissue-specific polypeptides performing as multi-valent linkages between tropocollagen molecules.[5] Although the concentration of natural cross-links in hides and skins are much lower compare to the artificial cross-linkers, they play a crucial role in the production of collagen fibrils.[5-7] Based on their mechanism of formation, the natural cross-links can be classified into immature (reducible on its -C=N- bond) and mature (non-reducible). Histidinohydroxylysinonorleucine (HHL) is a mature natural cross-link found specifically in skin, leading to denser packing of collagen molecules than tendon (without HHL),[8] supported by the less axial periodicity (D-period).[9] The

immature natural cross-links such as hydroxylysinoxidation products (HLNL), dihydroxylysinoxidation products (DHLNL) and histidinohydroxymerodesmosine (HHMD) were also found in skins and tanned leathers with comparable quantities.[7] Hence, with those natural cross-links constraining the intermolecular structure of collagen, a potentially improved tensile strength can be expected. Unfortunately, most of the immature natural cross-links are degraded into corresponding amino acid residues during processing. The instability of immature cross-links may result in the loss of many inherent qualities and properties of skin which needs to be reintroduced by virtue of artificial cross-linking (tanning). Insights into how natural/artificial cross-links affect the tensile strength of leather from the structural perspective will suggest a pathway to less intense leather tanning processes, by retaining the inherent cross-links.

X-ray and neutron scattering can provide useful information about changes in collagen structure.[10-12] Diffraction signals that originates from the long-range ordered structure of collagen can be detected by both X-ray and neutron sources. However, X-ray interacts with the electrons showing significant difference between untanned and mineral tanned leather samples. Neutron interacts with nuclei which enables the detection of the contrast between hydrogen and deuterium. Previous studies using neutron diffraction on deuterium-labelled cross-links in collagen with sodium borodeuteride established preliminary methods on observing specific signal differences of the labelled species.[13, 14] The structural effect of mineral artificial cross-linkers (tanning agents) on leather and its natural cross-links can also be studied using small-angle neutron scattering (SANS) based on the contrast variation of hydrogen and deuterium.

In this study, both static samples and *in situ* tensile measurements on SANS have been conducted providing evidence from a molecular and structural perspective on the contribution of tensile strength by natural cross-links and artificial cross-links.

2. Experimental methods

2.1. Materials

Raw lambskins were collected from a local tannery and transported to the New Zealand Leather & Shoe Research Association (LASRA), Palmerston North for processing in the pilot-tannery. Commercial reagents including, sodium hydroxide, disodium hydrogen phosphate, sodium borohydride, sodium borodeuteride, sodium sulphide, lime, ammonium chloride, hydrogen peroxide, sulphuric acid, sodium chloride, sodium bicarbonate, sodium formate and formic acid were used without further modification. Leather-specific reagents were obtained from their manufacturers, including Solvitose® from Avebe, Netherlands; Pancreol NB from Clariant, Germany; Intrapol LTN from the Shamrock Group Ltd; Chromosal® B (25% Cr₂O₃, 33% basicity) and Tanigan® PAK-N from Lanxess, Germany; Syntan HO from Smit & Zoon, Netherlands; Clarotan Mimosa vegetable extract from Tanac, Brazil; Polyol AK sulfated natural oil from Smit & Zoon, Netherlands, Sulphirol EG60 from Smit & Zoon, Netherlands; and Chromopol SG partially bisulfited natural oil from TFL, Germany.

2.2. Lambskin sample preparation

Skins of size 15cm*15cm were cut from the official sampling position (OSP) of the raw lambskins, and treated using excess (3 wt.% of the skin, same below) sodium borohydride (“reduced” or “R” in short) or sodium borodeuteride (“labelled” or “L” in short), while their counterpart from the skins were left untreated as control (“non-reduced” or “N” in short).

Samples were collected from the non-reduced, reduced and labelled raw skins (N1, R1 and L1) and the rest were processed to wet blue following ThruBlu chrome tanning. Samples were also collected at pickled stage (N2, R2 and L2) and wet blue stage (N3, R3 and L3) during the process, and kept at 4°C prior to SANS measurements.

An additional 10 extra lambskins were processed to wet blue in the same way as bioreplicates, with the OSP reduced by sodium borohydride at raw stage and the rest non-reduced.

Another group of 10 skins were cut into halves. Half of them were processed to wet blue using a modified chrome tanning method (a pickle-free tanning which retains natural cross-links), to compare with the other half tanned with the standard ThruBlu method. Then, the wet blue skins were processed to crust following LASRA standard post tanning method.

Detailed processing method is as follows:

Unhairing to pickling: Skins were painted using 10 wt.% sodium sulphide, 2.2 wt.% sodium hydroxide, 2.5 wt.% lime in 25g/L starch (Solvitose) solution, followed by hair removal and then liming with additional 80 wt.% of water. After washing, 2 wt.% ammonium chloride were used to delime and 0.12% enzyme (Pancreol NB) with 0.2% hydrogen peroxide were added to bate. After washing and fleshing,

2 wt.% sulphuric acid with 20 wt.% sodium chloride were added in 90 wt.% water to pickle the skins. In the modified tanning method, picking step and the neutralizing steps were skipped. Samples N2, R2 and L2 were collected at this stage.

Degreasing and Chrome tanning: 4 wt.% surfactant (Tetrapol LTN) and 5 wt.% sodium chloride were added in 50 wt.% water to degrease. Sodium formate and sodium bicarbonate were used to raise pH to 7.5-8.0 followed by an additional 100 wt.% water. Skins were washed thoroughly and 6% chrome (Chromosol B) were added to tan. Samples N3, R3 and L3 were collected at this stage.

Post tanning: In 100 wt.% water, skins were neutralised to pH 4.6-4.8 using Tanigan PAK-N, sodium formate and sodium bicarbonate followed by washing and retanning using 2 wt.% Syntan HO and 3 wt.% mimosa in 100 wt.% float. Skins were then fatliquored using 1 wt.% Chromopol SG, 1.5 wt.% Sulphirool EG60 and 3.5 wt.% Polyol AK, and finally fixed with formic acid to pH 3.6-3.8.

2.3. Small-angle neutron scattering (SANS)

Samples SANS experiments were performed on the QUOKKA instrument at the OPAL reactor, Australian National Science and Technology Organisation (ANSTO), Sydney, Australia. To cover the q -range of interest (from 0.001 to 0.4 \AA^{-1} , $q = (4\pi/\lambda)\sin\theta$), three different instrument configurations were used in this study:

- (1) sample-to-detector distances = 2 m, wavelength = 5.0 \AA , exposure time = 5 min;
- (2) sample-to-detector distances = 14 m, wavelength = 5.0 \AA , exposure time = 20 min;
- (3) sample-to-detector distances = 20 m, wavelength = 8.1 \AA , exposure time = 30 min.

The size of neutron beam was 20 mm in diameter for all measurements.

Samples were cut into squares with size of 10 mm \times 10 mm \times 2 mm (L \times W \times H) and kept hydrated in quartz cells (1 mm in thickness, 20 mm in diameter, Hellma, Germany) for *ex situ* measurements. Samples for *in situ* tensile measurements were cut into strips with size of 100 mm \times 10 mm \times 2 mm (L \times W \times H) and loaded onto the specially designed tensile stage (Figure 1). During tensile measurements, water was added onto the sample between the interval of each scan to keep its moisture level.

The SANS data were reduced using QUOKKA SANS reduction macros (Revision: 1842) in the Igor software package (version 6.3.7.2, Wavemetrics, USA). The reduced data were analysed using an in-house developed fitting software.[7] The relative diffraction peak intensities were calculated as $R_{ij} = A_i/A_j$, where A_i is the area of peak i .

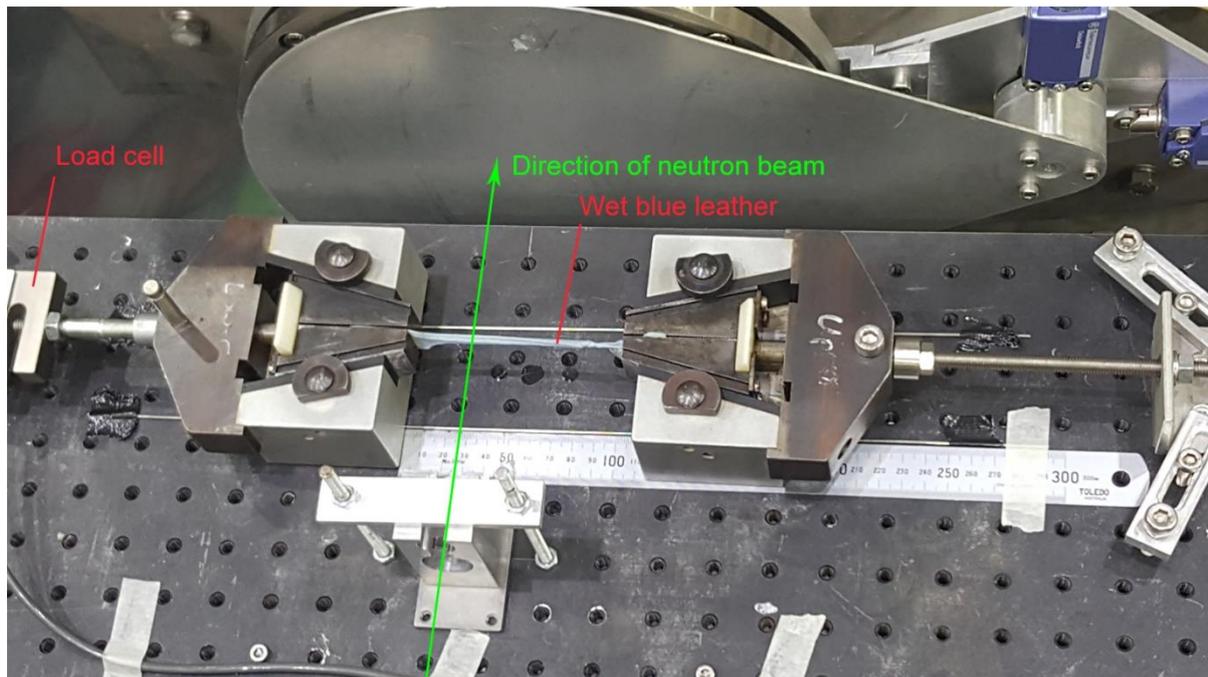


Figure 1. Tensile testing setup for wet skin and leather designed for operating at the SANS beamline.

2.4. Natural cross-link detection

The four natural cross-links (HLNL, DHLNL, HHL and HHMD) was detected following the same method as our previous study:[7] Briefly, skin samples were reduced using sodium borohydride and hydrolysed in 6 M HCl at 105 °C for 24 h. The hydrolysates were filtered and transferred to a CF-11 column, and eluted with butanol-water-acetic acid (4:1:1, v/v/v) to remove free amino acids. The natural cross-links were then eluted with water for analysis using LC-MS. The attachment of one deuterium onto each natural cross-link molecule in the labelled samples were also proven by its mass-to-charge ratio (m/z) detected by LC-MS.[15]

2.5. Tensile strength

The tensile strength was examined following the ISO procedures of leather tensile strength and percentage extension (ISO 3376) using an Instron tensometer, model number 4467. Three leather strips were collected perpendicular to the backbone from each of the replicates (120 in total) and were conditioned at 20 °C and 65% relative humidity for 72 h prior to the tensile tests.

3. Results and discussions

3.1. SANS scattering pattern of collagen in skins and leathers

SANS measurements of the skins and leathers showed characteristic diffraction rings and broad oscillation patterns which originate from the long-range ordered packing of collagen molecules (Figure 2).[16] The presence of the diffractions rings indicate the structural intactness of the collagen matrix (disordered collagen will not show diffraction peaks). The scattering patterns were then reduced to 1D by circular integration to analyse the two major peaks regarding their positions and intensities.

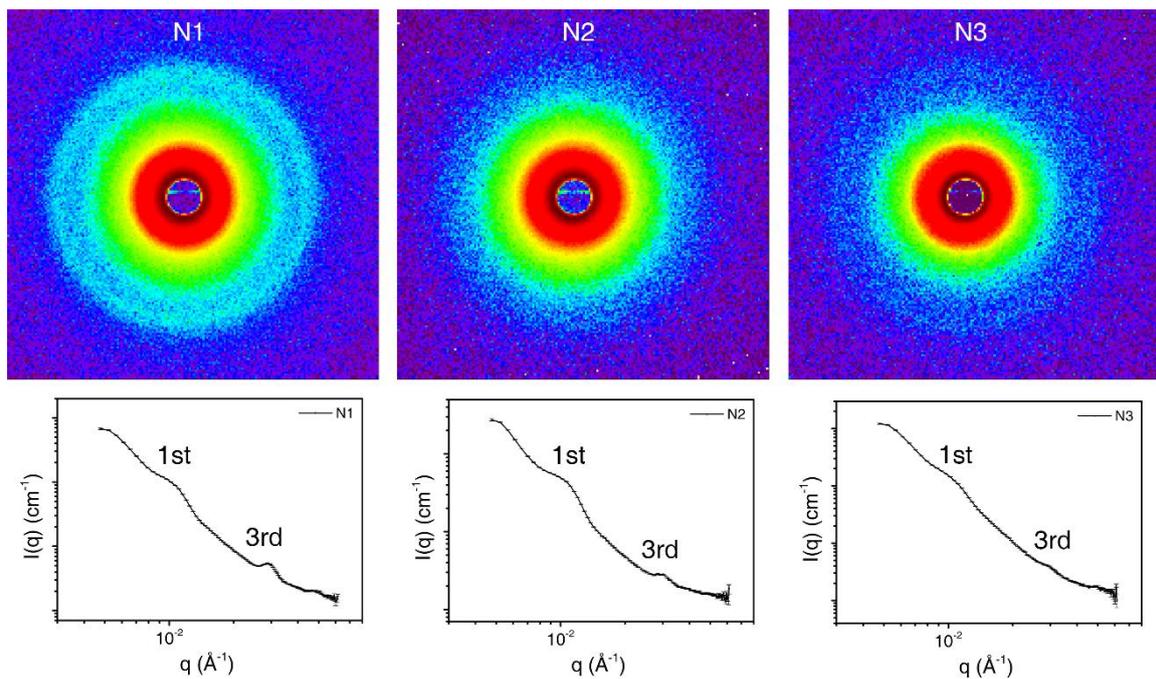


Figure 2. 2D SANS images from 14 m detector and the corresponding 1D q-plots by circular integration. N1, N2 and N3 stands for the non-reduced skins at raw, pickled and chrome tanned stages.[15]

The position of peaks on a q-plot indicates the axial packing periodicity (D-period) of collagen molecules within fibrils. The D-period is found to be 62-65 nm which is consistent to the previous results from SAXS studies;[7, 10, 11, 17] pickled samples were thinner and showed lower D-period compared to the raw and chrome tanned samples due to the condensed molecular packing in the collagen matrix.

3.2. Structural changes during tanning

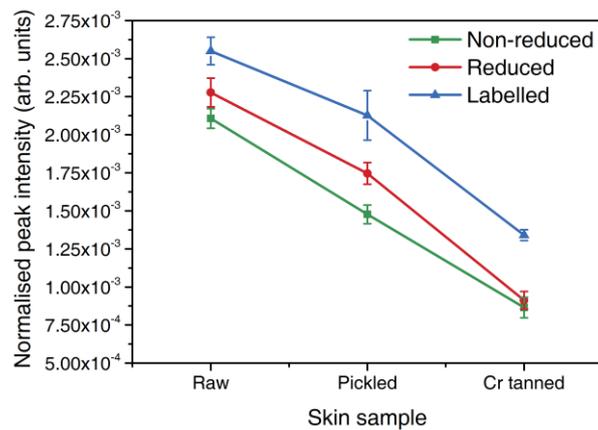


Figure 3. The normalised and averaged diffraction peak intensities of non-reduced, reduced and labelled raw skin, pickled skin and chrome tanned leather from SANS data analysis.[15]

Peak intensities showed significant changes during the tanning processes, which indicate that changes occur in the molecular level arrangement of collagen (Figure 3). Overall decrease in the 1st and 3rd order peaks were observed during pickling and chrome tanning for all the samples to a various extent. However, labelled samples showed increase in both 1st order peak and 3rd order peak highlighting the variation in contrast due to the introduction of deuterium into the matrix.

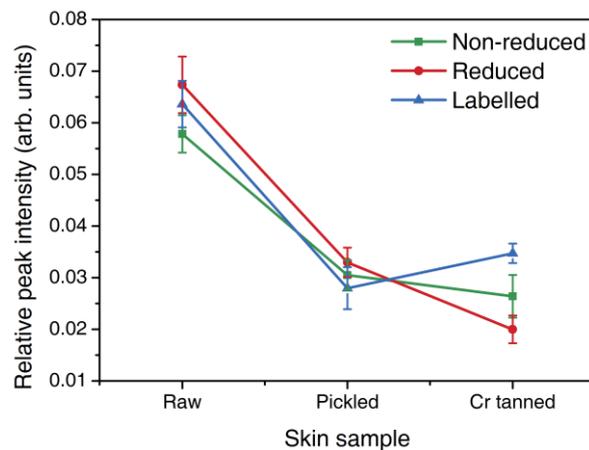


Figure 4. The relative peak intensity of 1st to the 3rd order diffraction peak of non-reduced, reduced and labelled raw skin, pickled skin and chrome tanned leather from SANS data analysis.[15]

These changes in diffraction peak intensities can be more explicitly shown as relative intensity (A_3/A_1) (Figure 4). All samples showed decreases in relative peak intensity from raw to pickled, however different trends were observed during chrome tanning. The relative peak intensity for reduced but unlabelled samples decreased significantly during tanning, while deuterium labelled leather shows an opposite trend during tanning. This opposite trend observed in the labelled samples indirectly confirmed the successful labelling of collagen natural cross-links by reduction using sodium borodeuteride. Such

contrast can be attributed to the alteration of hydration structure in the collagen matrix affecting the distribution of the deuterium/hydrogen nuclei in labelled/unlabelled samples.

3.3. Structural changes under stretching

The leather samples in this experiment were cut into strips perpendicular to the backbone, loaded horizontally and then stretched. SANS data collected before and during stretching clearly showed the direction of the collagen fibres in the leather samples (Figure 5). Generally, a group of collagen fibres that are aligned with each other will be observed as a pair of peaks on the intensity vs azimuthal angle (I vs ϕ) plot. Thus, the major part of the collagen fibres is aligned to around $\phi = 90^\circ$ and 270° before stretching (black), which matched with the direction of the backbone of the original leather. When stretching horizontally (red), the collagen fibres in leather were forced to align with the direction of the tensile load. This is observed as the presence of another pair of peaks at $\phi = 0^\circ$ and 180° .

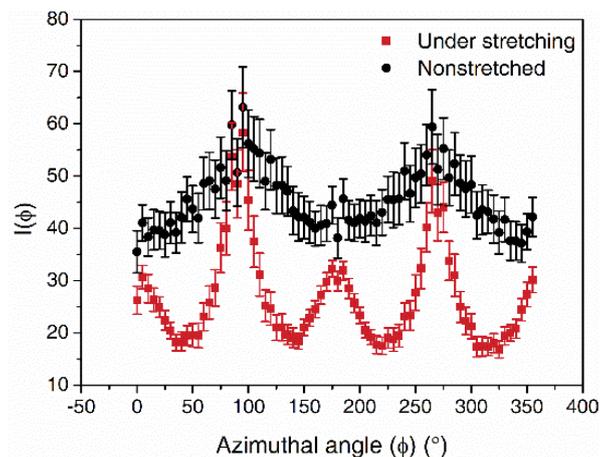


Figure 5. The intensity of the 1st order diffraction peak across all azimuthal angles around the beam centre for reduced chrome tanned sample (R3). Black: non-stretched; Red: at high tension (4.5N/mm²).

During stretching, the axial periodicity increased by 0.5-1.5 nm compared to the non-stretched samples. When it comes to the relative peak intensity, the effect of chrome tanning was found to dominate the changes of collagen structure under stretching (Figure 6). Both reduced and labelled samples showed unchanged relative peak intensity with increasing tensile force, indicating no significant impact from the labelled natural cross-links to the structure of collagen. On the other hand, the pickled skin showed a significant increase in relative peak intensity, confirming the major effect of chrome tanning to the stability of collagen molecular structure. Such increasing trend with tensile load may indicate the remaining flexibility of the collagen structure in the untanned skin.

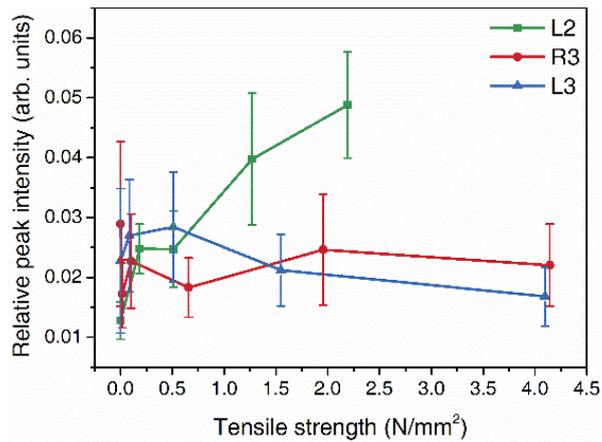


Figure 6. The relative peak intensity of 1st to the 3rd order diffraction peak of labelled pickled sample (L2), reduced chrome tanned sample (R3) and labelled chrome tanned sample (L3) from SANS data analysis.

3.4. Tensile strength

Three strips were collected perpendicular to the backbone from the OSP region of each leather sample (120 in total). Tensile strength tests were then conducted and a group of typical stress-strain curves were observed. As the strain increases, the tangent of the stress-strain curve first increases in the toe and heel regions, then remains constant in the linear elastic region until breakage. (Figure 7). Under stretching, the collagen fibrils can be straightened in the leather matrix, followed by the changes in the intermolecular spacing of collagen and the alignment of the collagen fibrils[18]. This rearrangement and increased order of the collagen fibrils were also confirmed during our *in situ* SANS experiments, in addition to the increased axial periodicity at high tensions.

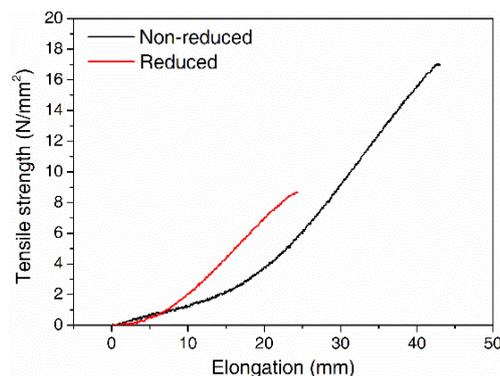


Figure 7. Stress-strain curves of non-reduced (black) and reduced (red) leather samples perpendicular to the backbone, at a strain rate of 100 mm/min.

While the reduction process stabilizes the natural cross-links in the collagen matrix, the leathers become stiffer and is supported by a quicker response to external force (Figure 7). This suggests a more constrained collagen structure with the presence of the retained cross-links. However, the reduction

process using sodium borohydride also caused damage to the surface of the leather samples, resulting in an overall lower tensile strength compare to the non-reduced leathers (Figure 8). The elongation at break were also found to be less than the untreated leather samples. These results suggest that a more benign method of stabilizing the natural cross-links is required instead of sodium borohydride.

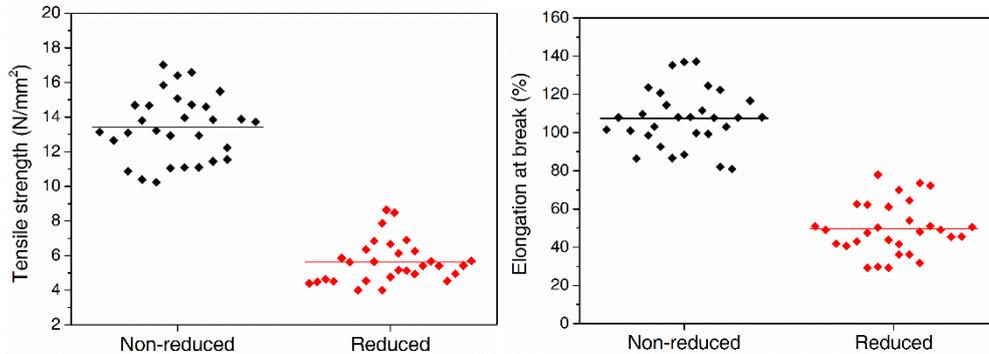


Figure 8. Scatter plot of the tensile strength and the elongation at break of the leather samples perpendicular to the backbone, with lines showing the average values. Black: standard LASRA ThruBlu leather; Red: Leather reduced with sodium borohydride prior to processing.

A moderate method of retaining natural cross-links by avoiding the picking step were applied to the leather tanning process to test its viability. However, after chrome tanning, the quantity of natural cross-links can decrease by 60% to 80% which makes their effects on the tensile strength remains minor:[15] the leather with natural cross-links were slightly stiffer shown by its lower average value of the elongation at break. The changes in tensile strength were also not significant. Such finding confirms the observations from the SANS experiments that, the macroscopic properties of the leather products is dominated by the artificial cross-linker (chrome) along with minimal contribution from the low concentration natural cross-links.

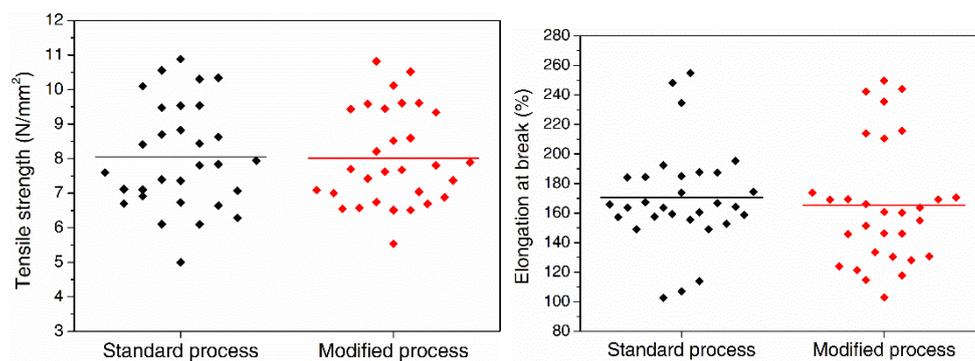


Figure 9. Scatter plot of the tensile strength and the elongation at break of the leather samples perpendicular to the backbone, with lines showing the average values. Black: standard LASRA ThruBlu leather; Red: modified chrome leather.

4. Conclusion

From the *in situ* tensile experiment with SANS, various structural events during leather stretching were observed from a molecular perspective. SANS results showed significant structural changes of collagen during the pickling step and the chrome tanning step. An obvious contrast between the labelled and unlabelled samples were observed especially during chrome tanning, suggesting a covalent cross-linking mechanism causing hydration structure changes which affects the distribution of deuterium nuclei in the collagen matrix. During stretching, the peak intensity indicates a comparably stiffer collagen structure in chrome tanned leather versus the untanned leather, highlighting the dominating role of covalent artificial cross-linking introduced during chrome tanning. Due to the lack of sufficient quantity compared to the artificial cross-linker, the natural cross-links in skins and leathers was not found to impart significant improvement in the tensile strength of the leather samples after chrome tanning.

5. Future work

This preliminary work using SANS on skins and leathers opens a novel perspective in leather research, providing molecular level insights during various events in leather processing and testing. Based on our observations from this study, future work would be conducted on the synthesis of new tannages as alternatives to chrome to reduce environmental impact while producing leather with similar properties. The use of a combination of pickling-free process and benign tanning agent to retain the intrinsic properties of leather will also be studied in the future. The variety amongst the properties of different leather will then be analysed using SAXS or SANS techniques.

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What makes leather stronger? A mechanistic study on the effect of natural/artificial cross-links on tensile strength using small-angle neutron scattering (SANS)

The ability to retain the intrinsic properties of hides and skins by stabilizing natural crosslinks is an exciting prospect for leather science. After selective labelling the crosslinks, a clear contrast between the labelled and unlabelled samples were observed especially during chrome tanning. This suggests that during the covalent cross-linking mechanism the hydration structure changes affecting the distribution of deuterium nuclei in the collagen matrix. SANS results also showed significant structural changes of collagen during the pickling step and the chrome tanning step. From the in situ tensile experiment with SANS, various structural events during leather stretching were observed from a molecular perspective.

I would like to thank the selection committee of the 2018 IULTCS/IUR Young Leather Scientist Grant for giving Yi Zhang the opportunity to conduct this work. The major findings can be summarized below.

- Natural crosslinks were selectively labelled using sodium borohydride. However, for the purposes of leather manufacturing a more benign process of crosslink stabilization is required.
- The presence of tanning agents such as chrome dominates over any properties from the natural crosslinks due to their low concentrations.
- Future studies will include the use of a more selective cross-link stabilization process, a pickle free process to preserve most of the natural cross-links and a benign tanning agent that will work alongside the intrinsic natural crosslinks.

Yours sincerely,

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