ACS Sustainable Chemistry & Engineering

Research Article

Cite This: ACS Sustainable Chem. Eng. 2018, 6, 7096−7104

pubs.acs.org/journal/ascecg

Revealing Molecular Level Indicators of Collagen Stability: Minimizing Chrome Usage in Leather Processing

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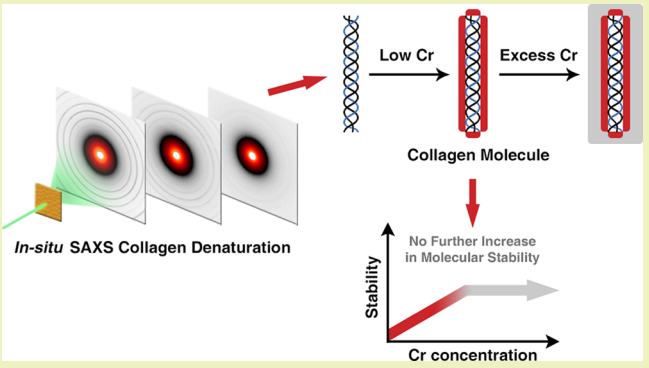
Supporting Information

ABSTRACT: Chromium(III) sulfate is extensively used in leather processing to stabilize the collagen molecules in hides and skins. Although its excess usage causes severe environmental pollution and health concerns, the role of chromium in stabilizing collagen still remains poorly understood. For the first time, by integrating a number of techniques, including real-time small-angle X-ray scattering, differential scanning calorimetry and natural cross-link analysis, we reveal crucial molecular-level indicators of collagen stability. The results indicate that collagen molecules achieve maximum molecular stability at concentrations as low as 1.8 wt % even if excess chromium (>3.7%) is introduced into the collagen matrix. At low concentrations (1.8% to 3.7%), the active amino acid residues are saturated via covalent bonding with chromium. Any excess chromium interacts purely non-covalently with the collagen molecule and, we propose, can be substituted by environment-friendly alternatives. Further, important natural cross-links, which are crucial in imparting mechanical strength, were observed to decrease with increasing chromium concentration, highlighting the adverse impact of chromium(III) sulfate on collagen matrix and the importance of identifying alternative cross-linking agents. Our findings provide tools which will enable the evaluation of greener tanning agents to facilitate a more sustainable future for the leather industry.



KEYWORDS: *Collagen structure, Denaturation, Chromium sulfate, In situ SAXS, Cross-link*

INTRODUCTION

Basic chromium(III) sulfate (BCS) is extensively used in conventional tanning to produce leathers with high hydrothermal stability and excellent organoleptic properties in a relatively short period of time.[1] However, conventional chrome tanning processes utilize 6% to 8% of BCS by weight relying on a concentration gradient to drive penetration, while an uptake of 40% to 70% is typical depending on the nature of animal hides and skins.[1−3] The poor uptake of BCS results in environmental stress and potential toxicity relating to hexavalent chromium (Cr VI) exposure,[3,4] leading researchers to reassess the case of chromium in modern tanning. Previous studies aimed to achieve satisfactory leather properties using less chromium with higher uptake by involving nanocomposites, pretanning agents, or novel processing methodologies.[2,5−8] However, the evaluation of properties remains superficial with lack of molecular level insights, hindering researchers from finding comprehensive solutions to sustainable chrome tanning. Considering that collagen is the primary structural element in the extracellular matrix in animal hides and skins, the heat resistance of leather against shrinkage was determined by the hydrothermal denaturation temperature (T_d) of collagen molecules.[9,10] However, such a commonly used indicator is based on heat transfer and lacks molecular level observations of collagen structural changes during denaturation, which is essential to understand the chromium−collagen reaction and for improving the sustainability of chrome tanning.

Collagen molecules are aligned in a quarter stagger structure, resulting in repeating gap/overlap regions within the fibrils.[11] Because of its ordered arrangement, molecular level information can be acquired by X-ray scattering techniques including diffraction, to measure the periodicity of the gap/overlap regions (*D*-period) and the size of collagen fibrils.[12−15]

Received: March 1, 2018
Revised: April 6, 2018
Published: April 20, 2018

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DOI: 10.1021/acssuschemeng.8b00954
ACS Sustainable Chem. Eng. 2018, 6, 7096−7104

Studying changes in these molecular level indicators during collagen denaturation process can reveal the mechanism by which chromium stabilizes collagen, and could lead to more efficient use of BCS during the tanning process. Further, natural cross-links in fibrillar collagen are known to play an important role in imparting mechanical strength in hides and skins.[16−18] To the best of our knowledge, the influence of chromium on these natural cross-links has yet to be studied.

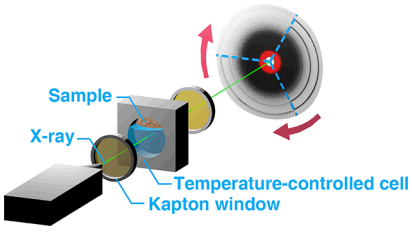
In this work, using synchrotron-based small-angle X-ray scattering (SAXS), we studied in real-time the denaturation of chromium stabilized fibrillar collagen in tanned leathers. We also studied the cross-section of leather using both *ex situ* SAXS and differential scanning calorimetry (DSC) to compare the molecular level indications (*D*-period and peak intensities) with the denaturation temperature (T_d). We investigated the effect of chromium sulfate on natural cross-links by isolating and quantifying them in tanned leathers. On the basis of the findings from this study, we have highlighted the possibility of using less chromium sulfate in leather processing to achieve similar performance to conventional systems and suggest a mechanism for collagen stabilization based on our results.

EXPERIMENTAL METHODS

Materials and Methods. *Sample Preparation.* Pickled ovine skins were treated following a conventional chrome tanning process with modifications.[14] First, pickled skins were fleshed, degreased and neutralized without using oxazolidine E (Zoldine ZE) for pretanning. The neutralized skins were then treated with different amounts (1%, 2.5%, 4.5%, 6% by weight) of BCS for 12 h. After fixation, the skins were rinsed and the tanning solutions collected for further analysis. Skin samples of size 0.5 cm × 0.5 cm × 3 mm (L × W × H) were cut out for the *in situ* denaturation experiments and 0.5 cm × 2 mm × 3 mm strips were cut for *ex situ* cross-section measurements. Detailed processing steps are described in the Supporting Information.

Small-Angle X-ray Scattering (SAXS). Isotropic and cross-sectional SAXS measurements were performed on beamline 23A1 at the National Synchrotron Radiation Research Centre in Hsinchu, Taiwan. Measurements of between 10 and 30 s, depending on the sample thickness, with an energy of 15 000 eV were performed. Scattered radiation was collected using a Pilatus 1 M detector located at a distance of 3.233 m from the sample. *In situ* experiments were carried out in the standard beamline temperature-controlled cell with the X-ray beam perpendicular to the skin surface (Scheme 1). *Ex situ* measurements were performed at room temperature (~25 °C) in the same sample cell. Wet samples were sealed in polyimide film (to prevent sample dehydration) and loaded onto the cell. The scattered intensity $I(q)$ is presented as a function of scattering vector, q, with $q = 4\pi \sin(\theta/2)$, where θ is the angle between incident and scattered radiation.

Scheme 1. Experimental Setup of *in Situ* SAXS Measurements



Differential Scanning Calorimetry (DSC). Differential scanning calorimetry (DSC) measurements were carried out using a Q2000 DSC (TA Instruments). Lyophilized samples were rehydrated overnight with DI water of same weight and then encapsulated in hermetically sealed aluminum pans (10 μL). All measurements were carried out over a temperature range of 30 to 120 °C under N_2 purge and a heating rate of 5 °C/min.

Scanning Electron Microscopy (SEM). Half-denatured samples were prepared by partially submerging a single strip of BCS tanned ovine leather by dipping into a water-glycerol (1:1, v/v) mixture at 120 °C for 3 min and leaving to dry under ambient conditions. A longitudinal cross-section through the non-denatured (unsubmerged) to the denatured (submerged) regions was made using a razor blade and then mounted onto a SEM pin stub such that the section was facing the electron beam. The specimen was sputter-coated with ~40 nm platinum and imaged using an FEI Nova NanoSEM 450 FE-SEM operating at 5 kV using a spot size of 3 and a working distance of 5 mm.

Natural Cross-Link Analysis. Natural cross-links in four biological replicates of skin and tanned leather (at each chromium concentration) were analyzed based on a previously published method.[19] Briefly, lyophilized samples were weighed and rehydrated in a phosphate buffered saline then reduced with sodium borohydride at 25 °C for 24 h. The reduction was quenched by adjusting the pH to 3.0 using acetic acid then reduced samples washed three times with water and lyophilized. The lyophilized reduced samples were hydrolyzed in 3 mL of 6 M HCl containing 3% phenol at 105 °C for 24 h and the resulting hydrolysates were then dried and rehydrated in 1 mL of water. Prior to extraction of the cross-links using CF-11 column, 10 μL was removed from each sample for hydroxyproline analysis. The remaining samples were subjected to CF-11 column and the water eluent contained the cross-links were then lyophilized and dissolved in 1 mL of water. Hydroxylysinonorleucine (HLNL), dihydroxylysinonorleucine (DHLNL), histidinohydroxylysinonorleucine (HHL) and histidinohydroxymerodesmosine (HHMD) concentrations were determined then normalized to the collagen content of the sample, which was calculated based on their hydroxyproline content.[20,21]

Chromium Analysis. Chromium concentration in the BCS tanned leather samples was measured by Varian SpectrAA 220FS Atomic absorption spectrophotometer and normalized to the collagen content in the leather samples. The amount of chromium present in the solution after the tanning process was also measured using AAS. Leather samples and tanning solutions were treated with an excess of concentrated nitric acid followed by a mixture of perchloric acid and sulfuric acid to solubilize chromium species. The solutions were then diluted and boiled again for 10 min to eliminate the residual oxidizing acids. After cooling, the solutions were further diluted and filtered before measurements. The measurements were conducted using an air/acetylene flame with a wavelength of 357.9 nm and slit width of 0.2 nm.

RESULTS AND DISCUSSION

SEM examination of BCS tanned leather at a chromium concentration of 5.8% (Figure 1A) revealed an open collagen fiber structure in the intact regions (top region of image) that gradually becomes closed and compact in the denatured regions. At room temperature (25 °C), the collagen fibers in the intact region are organized as groups of bundles in a relatively loose structure. High magnification SEM of the intact region (Figure 1B) shows collagen fibrils exhibiting typical collagen D-banding features.[15] Within a fibril, collagen molecules are aligned in a parallel staggered pattern resulting in repeating gap/overlap regions observed as the *D*-period.[11] On increasing the temperatures (Figure 1C), a distorted fibril morphology lacking D-banding with an apparent increase in fibril diameter was observed. Denaturation of collagen fibrils in a hydrated environment is known to occur due to the breaking of hydrogen bonds and cross-links (natural and artificial).[22−24] An apparent increase in the fibril diameters accompanied by a decrease in their length is observed, resulting in the macroscale

DOI: 10.1021/acssuschemeng.8b00954
ACS Sustainable Chem. Eng. 2018, 6, 7096−7104

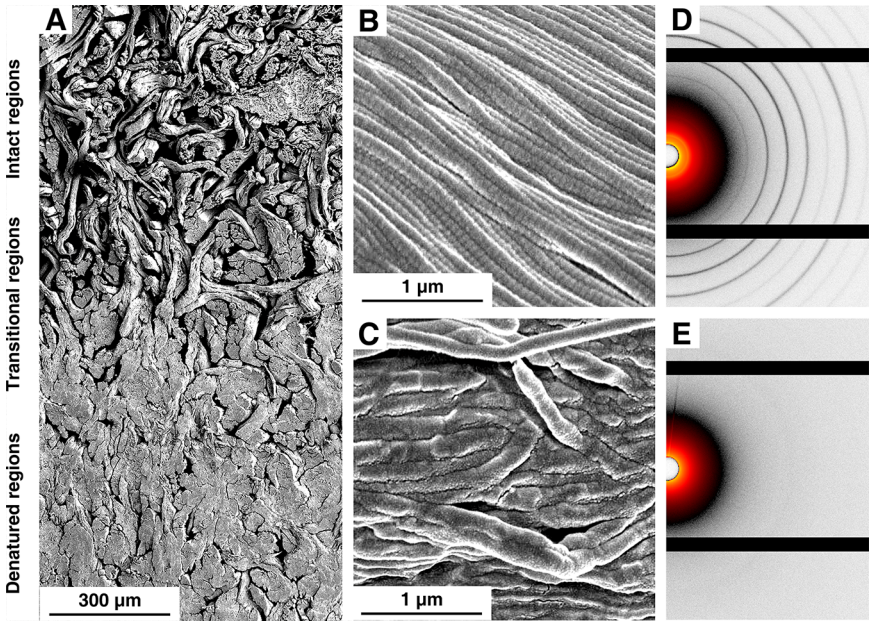


Figure 1. Secondary electron micrographs of a half-denatured chromium tanned leather sample at (A) low magnification across the section from the intact (top of image) through to the transitional (middle) and denatured (bottom) regions; and at high magnification focusing on the (B) intact and (C) denatured regions. SAXS scattering patterns acquired from (D) intact and (E) denatured regions.

Table 1. Relationship between Collagen *D*-Periodicity and the Chromium Concentration in Untanned Skin (0%) and BCS Tanned Leather (1.8% to 6.3%) samples

Cr (wt %)[a]	0% (untanned)	1.8% (±0.1)	3.7% (±0.3)	5.8% (±0.1)	6.3% (±0.1)
D-period (nm)	63.9 ± 0.2	64.8 ± 0.1	64.8 ± 0.1	64.7 ± 0.1	64.6 ± 0.1

[a]Chromium concentrations were normalized to collagen content in each sample.

shrinkage of the collagen fibers.[25] SAXS measurements of the intact region (Figure 1D) showed intense diffraction rings characteristic of collagen treated with BCS tanning agent, which improves both the long-range order as well as the electron density contrast of the collagen. By contrast, denatured chrome tanned leather is characterized by the absence of visible diffraction rings (Figure 1E) resulting from collagen denaturation, featuring disordered intermolecular arrangement within fibrils.[26,27]

***Ex Situ* Isotropic SAXS Results of Untanned and Tanned Samples.** From *ex situ* SAXS results, an increase in the *D*-period from 63.9 to 64.8 nm (Table 1) was observed with an increase in the chromium concentration from 0% to 1.8%. This was also indicated by the slight shift in the position of all diffraction peaks to lower q (dashed line, Figure 2). By increasing the chromium concentration from 1.8% to 6.3%, no further shifts in diffraction peaks were observed, indicating the *D*-period was unchanged. These observations were in good agreement with Maxwell et al., who observed similar *D*-period changes on chrome tanned bovine hide.[12] On the basis of the reactivity of chromium species with aspartic and glutamic acid side chains,[9] we suggest that all the telopeptidyl aspartic and glutamic acid residues of collagen are covalently bound to chromium at a concentration of 1.8%. Subsequently, at higher chromium concentrations, further reactions in the telopeptidyl regions are no longer possible, with no significant changes in collagen *D*-period being identified.

Substantial variations in the overall peak intensity were observed on untanned skin and BCS tanned leathers at different chromium concentrations (Figure 2). In this study, the

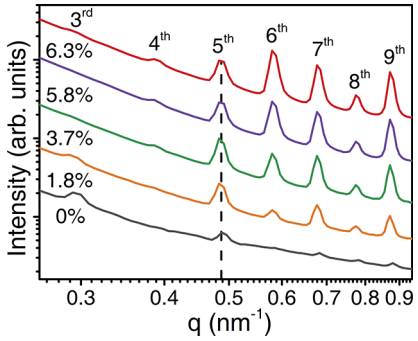


Figure 2. *Ex situ* SAXS data showing differences in scattering intensity between untanned skin (0%) and BCS tanned leather samples with 1.8%, 3.7%, 5.8% and 6.3% of chromium normalized to the collagen content. Selected diffraction peaks corresponding to $q = 2\pi n/D$ where n is the peak order and D is the *D*-period, are labeled.

intensity of the third order peak initially decreased with increasing chromium concentration from 0% to 1.8% and disappeared at 3.7%, followed by the reappearance at 6.3%. The intensity of the fifth order peak gradually increased at lower chromium concentration from 0% to 3.7% and slightly decreased at high concentration (5.8% to 6.3%). The sixth to ninth order peak intensity constantly increased with raised chromium concentration (0% to 6.3%) (Figure S1). Previous studies have shown that the intensity of the odd order diffraction peaks decreased while the even order diffraction peaks (particularly the sixth order) increased following the dehydration of fibrillar collagen.[28,29] However, the relative

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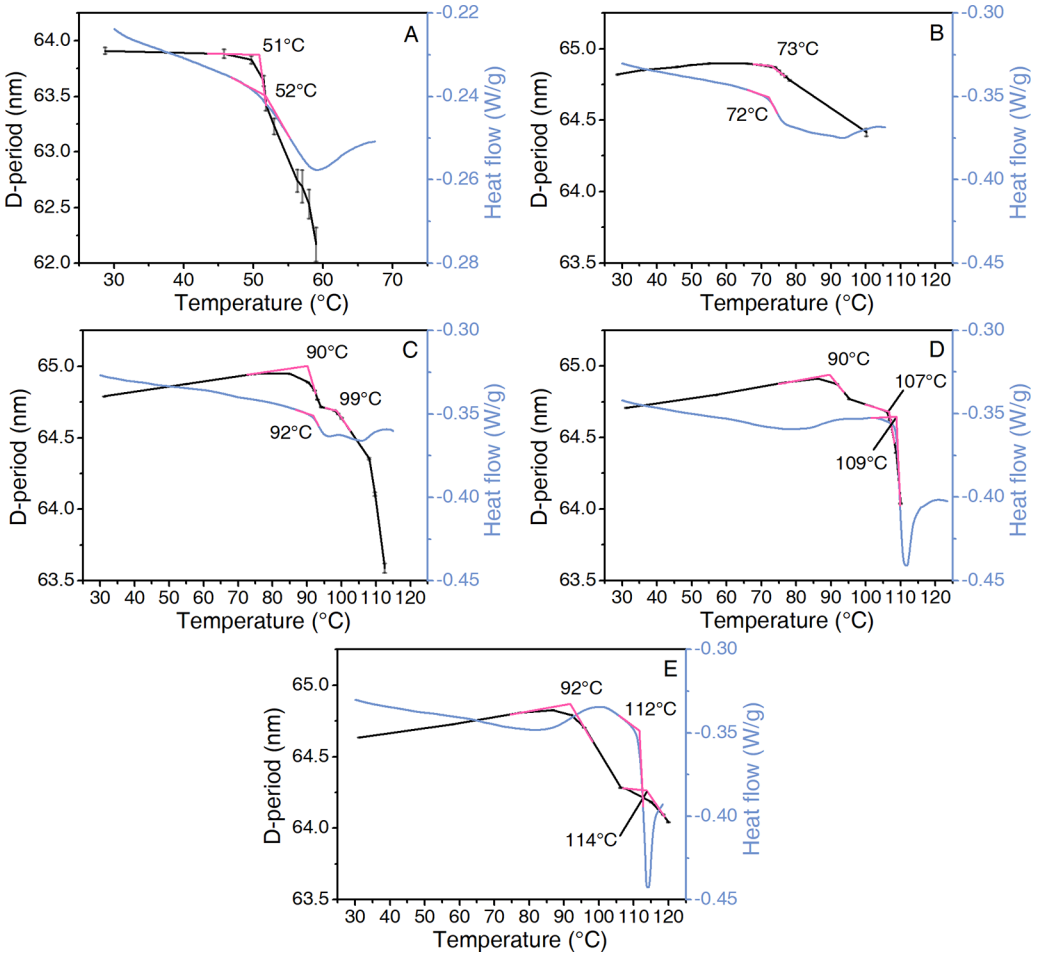

Figure 3. Changes in *D*-period obtained from *in situ* SAXS data analysis and heat flow changes from DSC results (blue lines) during the denaturation of (A) untanned skin and tanned leather samples with (B) 1.8%, (C) 3.7%, (D) 5.8% and (E) 6.3% of chromium normalized to the collagen content. The onset temperatures (T_{onset}) of the *D*-period during denaturation and the corresponding denaturation temperatures (T_d) of each sample are calculated and labeled alongside each curve.

intensity of the fifth order to the third order decreased or remained constant upon dehydration of untanned collagen,[28−30] unlike the increase we observed during this study and other previously reported SAXS results on BCS tanned collagen.[14] Since the introduction of chromium species is the major variable between the untanned and tanned samples, this mismatch is most likely due to the covalent bonding effect of chromium with collagen. Therefore, the relative intensity can be used as an indicator of chromium reacting with collagen. Furthermore, it has been shown previously that the introduction of metallic species into collagen matrices contributes to the electron density of collagen, resulting in a noticeable increase in the overall peak intensity.[12,28,31,32]

Based on these observations, we speculate that initially the interaction of chromium species with the telopeptidyl and helical aspartic and glutamic acid residues produce covalent bonds with the collagen. This interaction, in addition to the enhanced electron density contrast from the inorganic Cr species, resulted in a gradual decrease in the intensity of the third order peak and an increase in intensity of the others (fourth to ninth order). After all aspartic and glutamic residues are covalently bound by chromium species, non-covalent interactions such as hydrogen bonding on charged amino acid residues of collagen with the aquo ligands and hydroxyl bridges of chromium complexes will dominate further reactions,[33] forming a layer of chromium species depositing around the collagen molecules.[34] Such interactions cause the dehydration of the collagen molecule while continuing to enhance its electron density contrast. This resulted in the relatively faster increase of the sixth order peak at chromium concentration higher than 3.7% (Figure S1A), along with the reappearance of the third order peak at chromium concentration of 6.3% (Figure S1B).

***In Situ* Isotropic SAXS and DSC Results of Untanned and Tanned Samples.** Chromium species stabilize the collagen structure through the formation of covalent bonds with amino acid side chain groups particularly aspartic and glutamic acid.[9] However, to the best of our knowledge, the relationship between different chromium concentrations and collagen structural changes at the molecular level and their effect on denaturation temperature (T_d) is, so far, not fully understood. In this study, we used an *in situ* approach to investigate the role of chromium in stabilizing collagen structure, which is crucial in optimizing its use in leather production. By using a temperature-controlled cell on the SAXS beamline we were able to study real-time changes in the collagen structure of BCS tanned leather during the denaturation process.

The *D*-period changes obtained from the SAXS results during collagen denaturation are plotted with the correspond-

DOI: 10.1021/acssuschemeng.8b00954
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ing DSC heat flow changes of chromium concentrations (0%, 1.8%, 3.7%, 5.8% and 6.3%) (Figure 3A–E). The normalized fifth order peak intensity is also plotted against temperature for each sample, as an indicator of overall changes in intensity during collagen denaturation, due to it being measurable at all chromium concentrations (Figure 4). The *D*-period of the

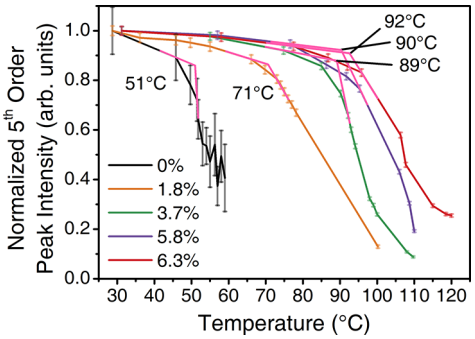


Figure 4. Changes of the fifth order peak intensity from *in situ* SAXS data analysis, normalized to the starting material in each experiment. The onset temperatures (T_{onset}) of the fifth order peak intensity during denaturation of each sample are calculated and labeled alongside each curve.

untanned sample remained constant at 63.9 nm until it drastically decreased at its T_d, which was accompanied by a sudden loss in peak intensity (Figure 3A). The triple helical structure of collagen and fibrils in register are stabilized by hydrogen bonds and natural cross-links, respectively.[18] Heating can disrupt these bonds and uncoil the collagen triple helices, resulting in a diminished gap/overlap region observed as a sudden decrease in *D*-period. This leads to the denaturation of collagen along with a loss in long-range order of the fibrils, as can be seen from the drop in the diffraction peak intensity.[35] We noted that the onset temperature (T_{onset}) of changes in *D*-period and T_d in the untanned sample overlapped, indicating that uncoiling of collagen triple helix and disrupting of the collagen quarter stagger pattern are occurring simultaneously.

In contrast, a slight increase in *D*-period was observed prior to its T_{onset} for tanned samples (Figure 3B–E). Heating causes conformational changes of the collagen triple helix,[36] and could affect the arrangement of the chromium layer around the collagen molecule resulting in a slight increase in our *D*-period observations. Also, at increasing chromium concentrations from 1.8% to 6.3%, the lowest T_d in each sample, increased from 72 to 112 °C (Figure 3B–E). The broad endothermal peaks in samples at low chromium concentration of 1.8% and 3.7% and the sharp peaks in higher chromium concentration samples at 5.8% and 6.3%, highlighted the non-uniform T_d throughout the skin at low chromium concentration during the tanning processes (Figure 5C). At a chromium concentration of 1.8% (Figure 3B), the T_{onset} of *D*-period and the lowest T_d overlapped at 73 and 72 °C, with an associated drop in the peak intensity at around 71 °C. At higher chromium concentrations of 3.7% (Figure 3C), apart from the first T_{onset} of *D*-period at 90 °C, a second T_{onset} of *D*-period was identified at 99 °C. The first T_{onset} of *D*-period overlaps with the T_{onset} of peak intensity at 89 °C, as well as the lowest T_d at 92 °C. Instead, the second T_{onset} of *D*-period was observed to mirror the higher T_d through the cross-section of the sample around 100 °C (Figure 5C). Similar mismatches were found when chromium concentration was raised to 5.8% and 6.3% (Figure

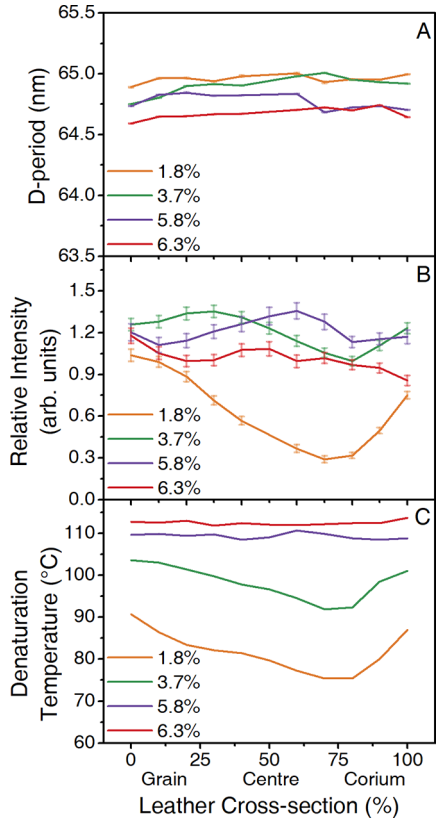


Figure 5. Cross-sectional results showing (A) *D*-period and (B) relative diffraction peak intensity of fifth and third order peaks obtained from SAXS data analysis; (C) denaturation temperatures (T_d) from DSC analysis through the BCS tanned leather samples at different chromium concentrations.

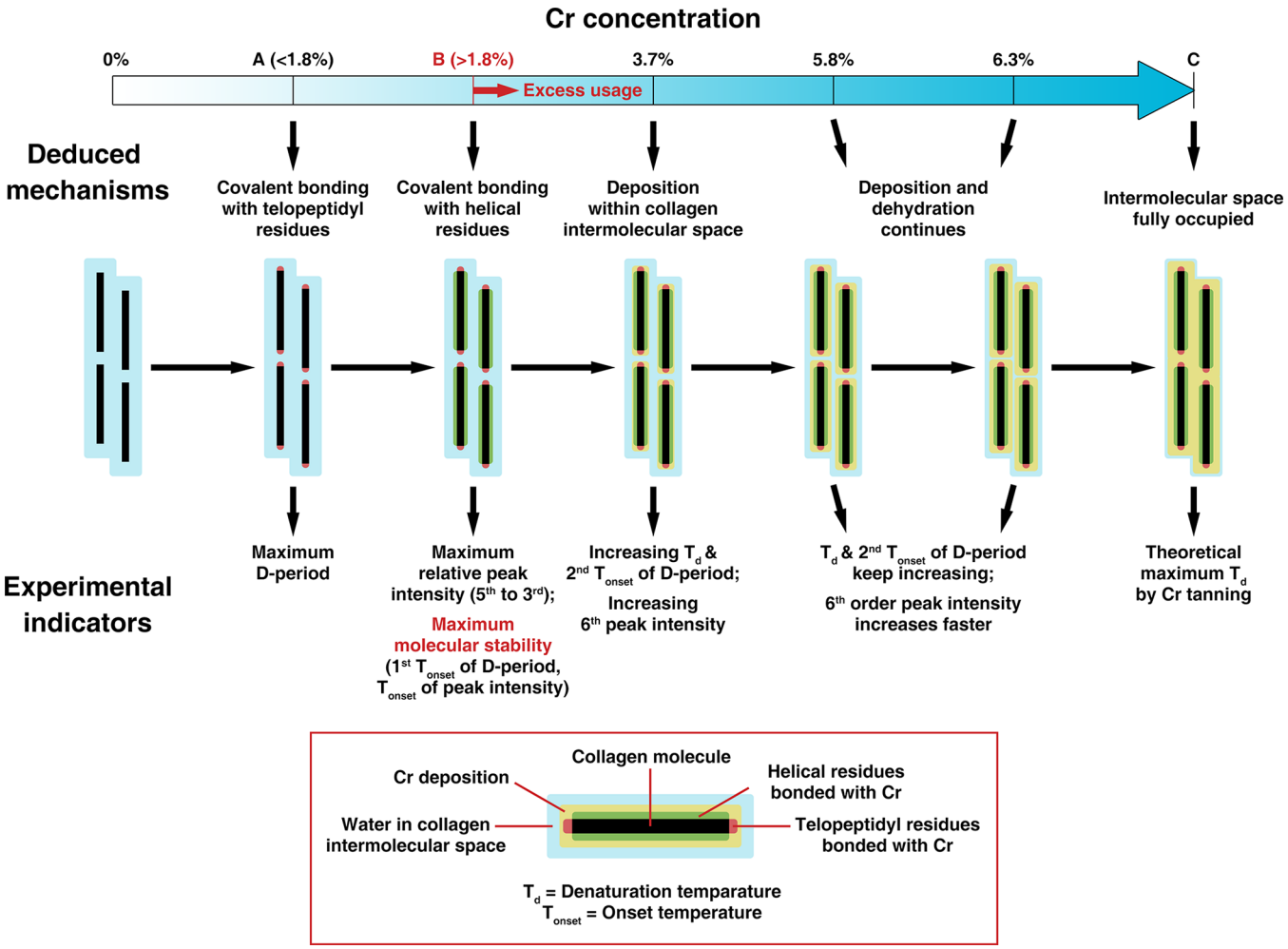
3D,E), despite the uniform T_d. While the first T_{onset} of *D*-period remained unchanged at around 90 °C, to overlap with the T_{onset} of peak intensity, the second T_{onset} of *D*-period again corresponded to the T_d at around 110 °C.

We hypothesize the reasons for the mismatches among the T_{onset} are as follows:

1. The decrease in peak intensity at T_{onset}, which overlaps with the first T_{onset} of *D*-period is a direct indicator of the loss of long-range order in collagen. The intermolecular staggering order can be disrupted by the conformation changes (pre-uncoil) within the collagen molecules at the relatively free telopeptidyl regions and other thermally labile regions.[36,37] This metastable triple helical structure will be destroyed by any further temperature ramps and completely uncoil. For tanned samples, the labile regions are speculated to be where the less covalent chromium–collagen bonds are formed. Once the active residues are fully bonded with chromium, the thermal stability of the labile region within a single collagen molecule reaches the maximum for BCS tanning (around 90 °C). This also explains the features in the 5.8% and 6.3% samples that simultaneously showed up at the first T_{onset} of *D*-period, which possibly implies a thermodynamically stable pre-uncoiled stage for the stabilized collagen.
2. The T_d that overlaps with the second drop of *D*-period coincides with the complete uncoiling of collagen and is associated with the collapse of the intermolecular structure. As discussed above, after occupying all the

DOI: 10.1021/acssuschemeng.8b00954
ACS Sustainable Chem. Eng. 2018, 6, 7096–7104

Scheme 2. Mechanistic Model of the Reaction between Chromium and Collagen at Different Concentrations, Deduced from the Molecular-Level Experimental Indicators



active sites for covalent bonding, chromium species will then interact with collagen via non-covalent mechanisms such as hydrogen bonding and deposition.[33,34] Previous studies suggested that non-covalent interactions such as coating and hydrogen bonding can affect the thermal stability of collagen.[15,38,39] On the basis of these observations, we postulate that the hydrogen bonding and deposition on the collagen molecules and fibrils respectively, could confine the uncoiling of peptide chains, leading to the higher apparent T_d of collagen.

***Ex Situ* Cross-Sectional SAXS and DSC Results of Tanned Samples.** Skin cross-sections can be conveniently subdivided into grain, center and corium regions. Collagen fiber arrangement and density varies among different regions. Because of the non-uniform cross-sectional structure of the skins, to ensure the subsequent leather products meet the minimum requirement of hydrothermal stability throughout the cross-section, the penetration of chromium species needs to be taken into consideration for optimizing the tanning processes. Thus, to further investigate the optimum concentrations at which chromium stabilizes collagen, DSC measurements through the cross-section of BCS tanned leather samples were performed for comparison with the cross-sectional scans using SAXS. The resulting *D*-period, relative intensity of the fifth and the third order peak and T_d are plotted in Figure 5. The relative intensity of cross-sectional scans was calculated relative to the isotropic scans of untanned skin (identified as 0) and BCS tanned leather at chromium concentration of 6.3% (identified as 1). Fractional values from 0 to 1 denote the extent of the change from one to the other.

The *D*-period was found to be fairly constant throughout the cross-sections of all the BCS tanned samples containing the different concentrations of chromium (Figure 5A). This suggests that even at the lowest concentration in this study (1.8%), the number of chromium species that penetrated all the way through the skin is sufficient to exhaust the telopeptidyl aspartic and glutamic acid residues, producing uniform *D*-period changes throughout the tanned leather.

The relative peak intensity, on the other hand, gave varying results for the different chromium concentrations (Figure 5B). While the values were comparatively constant at a concentration of 3.7% and above, a U-shaped curve was observed at 1.8%. As an indicator of the covalent bonding of chromium with collagen, this clarifies that the aspartic and glutamic acid residues of collagen in samples with chromium concentration equal to or higher than 3.7% were completely exhausted. Interestingly, we found that when the chromium concentrations increase from 3.7% to 6.3%, the relative intensity throughout the cross-section stops increasing and instead decreases with increasing chromium concentration. At low chromium

DOI: 10.1021/acssuschemeng.8b00954
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concentration, the formation of chromium−collagen covalent bonds plays a dominant role in the change in relative intensity. However, at higher concentration the dehydration effect observed in the collagen molecule plays a greater role in changing the relative intensity. This suggests that using an excess of BCS tanning agent oversaturates the collagen matrix with chromium species, without imparting any intramolecular stability to the collagen matrix via covalent bonding.

The T_d values measured through the cross-section were observed to follow a similar mechanism (Figure 5C). Uniform values throughout the leather samples were observed at a chromium concentration of 5.8% and 6.3%, but samples at 3.7% showed a gradual decrease in T_d from the grain and corium surfaces to the center, indicating less non-covalent interactions between chromium and the collagen molecules. On the basis of the relative intensity of the sample at chromium concentration of 1.8%, we observed a non-uniform T_d with a maximum value of 90 °C on the grain surface. It is important to note that at this point, the relative intensity is around 1.0, i.e., the isotropic value of the oversaturated 6.3% sample. This shows that the covalent chromium−collagen bonding finishes at this concentration (not measured) and would be between 1.8% and 3.7%. This is in agreement with the *in situ* denaturation results, confirming the role of covalent bonding of chromium species with collagen and its upper limit on stabilizing the intramolecular structure of collagen.

To summarize the role of chromium on affecting the collagen structure at different concentration, a mechanistic model of collagen structure is demonstrated based on the observed molecular level indicators (Scheme 2). At extremely low chromium concentration (A < 1.8%), chromium species form covalent bonds with telopeptidyl active sites causing the expansion of the axial staggering gaps. The saturation is indicated by the maximizing of the D-period. At slightly higher chromium concentration (1.8% < B < 3.7%), chromium species covalently occupied the active sites in both the telopeptidyl and helical regions, resulting in the maximized relative intensity of the fifth and the third order peaks. The intramolecular stability of collagen thereby reaches its upper limit using BCS tanning, revealed by the unchanged first T_{onset} of D-period and peak intensity at 90 °C. Upon exhaustion of the active covalent sites, the relative intensity of the fifth and the third order peaks would be reduced slightly, indicating the dominance of non-covalent deposition. Meanwhile, the intensity of the sixth order peak starts to increase at a higher rate, confirming the dehydration effect due to chromium deposition and the displacement of water. The non-covalent interaction only contributes to the apparent denaturation temperature (T_d), which is supposed to stop once the intermolecular space is fully occupied.

Increased Economic Viability of Chrome Tanning. The leather industry plays a prominent role in the global economy with the sustainability of leather manufacturing largely affected by chemical usage during the tanning process. Based on our mechanistic model, BCS usage can be reduced from 6% to 2−3% to reach satisfactory tanning, with a proven improvement on percentage uptake of chromium by the leather matrix (Figure S2). This will improve the economic sustainability of the leather industry. According to reports, approximately 1.6 million chrome tanned bovine hides were produced throughout New Zealand in 2017.[40] Based on the average wet weight of 25 to 30 kg per piece of hide, the saving from halving BCS usage can be estimated to be more than 5 million US dollars per annum.

Natural Cross-Link Analysis of Untanned and Tanned Samples. The natural cross-links in collagen are known to contribute to the mechanical strength[18] of skins and, thus, the ultimate leather properties. The results from natural cross-link analysis showed a significant difference in untanned and tanned samples with increasing chromium concentration (Figure 6).

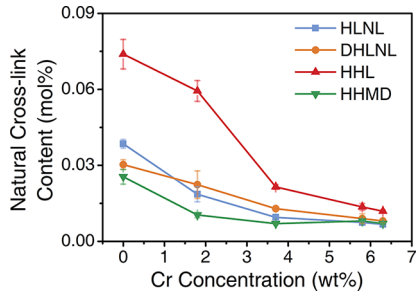

Figure 6. Natural cross-link content in untanned and tanned samples. Chromium and natural cross-link concentrations were both normalized to collagen content in each sample. (Pearson's r: HLNL = 0.915, DHLNL = 0.965, HHL = 0.971, HHMD = 0.805, p-value <0.05 for each of the natural cross-link).

All natural cross-link concentrations were normalized to the collagen content, which was measured from the hydroxyproline (Hyp) content in the sample.[20,21] Detailed calculations are shown in the Supporting Information (Table S2).

The highest amount of all types of natural cross-links were observed in the untanned skins, which decreased significantly during tanning with increasing chromium concentration. To the best of our knowledge, this is the first time that natural cross-links have been isolated and quantified in tanned leather samples. Although the mechanism of the decrease in natural cross-link contents remains unclear, we speculate that chromium interferes with the natural cross-links by reacting with collagen in the telopeptidyl regions, resulting in a diminishing amount of intact natural cross-links. Lower amounts of natural cross-links in the collagen of raw skins were observed to have a direct relationship to poor mechanical strength in the subsequent final leather product (data not shown). These adverse effects clearly emphasize the disadvantage of excess use of BCS in tanning. Therefore, alternative cross-linking agents which do not interfere with the natural cross-links are expected to be more efficient than chromium(III) based BCS tanning agent, and retain more strength.

CONCLUSIONS

From the molecular level observations and key indicators, we gained fundamental knowledge that helps distinguish the different events during conventional BCS tanning. Chromium concentrations higher than 3.7% are identified as excessive, because at such concentrations the active amino acid residues are fully exhausted by the covalent bonding with chromium. Further, non-covalent interaction only contributes to the apparent denaturation temperature (T_d), due to a dehydration effect. This suggests an upper limit of stabilization on collagen via BCS tanning. Additionally, the natural cross-links that contribute to the mechanical strength were diminished with increasing chromium concentration. This points to another disadvantage of BCS tanning, especially when used in excess.

DOI: 10.1021/acssuschemeng.8b00954
ACS Sustainable Chem. Eng. 2018, 6, 7096−7104

Both findings propose a feasible reduction of BCS usage, which can lead to a cost deduction of 5 million US dollars per annum, improving the economic sustainability of the leather manufacturing industry. More importantly, these key indicators establish a clear guide to modifying tanning processes and enable us to evaluate the efficiency of other environment-friendly alternative cross-linkers at the molecular level. This is essential to achieve satisfactory collagen stability and the requisite organoleptic properties of the final leather products, and subsequently, a pathway to a more sustainable future for leather processing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b00954.

Detailed experimental procedures, SAXS data analysis, normalized SAXS peak intensity plots, table of hydroxyproline and collagen content (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

S.P., Y.Z., R.N. and G.H. thank the Ministry of Business, Innovation and Employment (MBIE) for providing funding through grant LSRX-1301. Portions of this work was conducted on the SAXS beamline at NSRRC, Taiwan. Part of this research used the facilities at the Electron Microscope Unit at UNSW.

■ REFERENCES

(1) Covington, A. D. *Tanning chemistry: the science of leather*; Royal Society of Chemistry, 2009.
(2) Sundar, V.; Rao, J. R.; Muralidharan, C. Cleaner chrome tanning—emerging options. *J. Cleaner Prod.* **2002**, *10* (1), 69−74.
(3) Sreeram, K.; Ramasami, T. Sustaining tanning process through conservation, recovery and better utilization of chromium. *Resour., Conserv. Recycl.* **2003**, *38* (3), 185−212.
(4) Saha, R.; Nandi, R.; Saha, B. Sources and toxicity of hexavalent chromium. *J. Coord. Chem.* **2011**, *64* (10), 1782−1806.
(5) Sundarapandiyan, S.; Brutto, P. E.; Siddhartha, G.; Ramesh, R.; Ramanaiah, B.; Saravanan, P.; Mandal, A. Enhancement of chromium uptake in tanning using oxazolidine. *J. Hazard. Mater.* **2011**, *190* (1−3), 802−809.
(6) Lyu, B.; Chang, R.; Gao, D.; Ma, J. Chromium footprint reduction: nanocomposites as efficient pretanning agents for cowhide shoe upper leather. *ACS Sustainable Chem. Eng.* **2018**, *6* (4), 5413−5423.
(7) Ma, J.; Lv, X.; Gao, D.; Li, Y.; Lv, B.; Zhang, J. Nanocomposite-based green tanning process of suede leather to enhance chromium uptake. *J. Cleaner Prod.* **2014**, *72*, 120−126.
(8) Morera, J. M.; Bartolí, E.; Chico, R.; Solé, C.; Cabeza, L. F. Minimization of the environmental impact of chrome tanning: a new process reusing the tanning floats. *J. Cleaner Prod.* **2011**, *19* (17−18), 2128−2132.
(9) Covington, A. D. Modern tanning chemistry. *Chem. Soc. Rev.* **1997**, *26* (2), 111−126.
(10) Schroepfer, M.; Meyer, M. DSC investigation of bovine hide collagen at varying degrees of crosslinking and humidities. *Int. J. Biol. Macromol.* **2017**, *103*, 120−128.
(11) Petruska, J. A.; Hodge, A. J. A subunit model for the tropocollagen macromolecule. *Proc. Natl. Acad. Sci. U. S. A.* **1964**, *51* (5), 871−876.
(12) Maxwell, C. A.; Smiechowski, K.; Zarlok, J.; Sionkowska, A.; Wess, T. J. X-ray studies of a collagen material for leather production treated with chromium salt. *J. Am. Leather Chem. Assoc.* **2006**, *101*, 9−17.
(13) Maxwell, C. A.; Wess, T. J.; Kennedy, C. J. X-ray diffraction study into the effects of liming on the structure of collagen. *Biomacromolecules* **2006**, *7* (8), 2321−2326.
(14) Zhang, Y.; Ingham, B.; Cheong, S.; Ariotti, N.; Tilley, R. D.; Naffa, R.; Holmes, G.; Clarke, D. J.; Prabakar, S. Real-Time Synchrotron Small-Angle X-ray Scattering Studies of Collagen Structure during Leather Processing. *Ind. Eng. Chem. Res.* **2018**, *57* (1), 63−69.
(15) Zhang, Y.; Ingham, B.; Leveneur, J.; Cheong, S.; Yao, Y.; Clarke, D. J.; Holmes, G.; Kennedy, J.; Prabakar, S. Can sodium silicates affect collagen structure during tanning? Insights from small angle X-ray scattering (SAXS) studies. *RSC Adv.* **2017**, *7* (19), 11665−11671.
(16) Bailey, A. J.; Paul, R. G.; Knott, L. Mechanisms of maturation and ageing of collagen. *Mech. Ageing Dev.* **1998**, *106* (1−2), 1−56.
(17) Puxkandl, R.; Zizak, I.; Paris, O.; Keckes, J.; Tesch, W.; Bernstorff, S.; Purslow, P.; Fratzl, P. Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Philos. Trans. R. Soc., B* **2002**, *357* (1418), 191−197.
(18) Fratzl, P. *Collagen: structure and mechanics*; Springer Science & Business Media, 2008.
(19) Naffa, R.; Holmes, G.; Ahn, M.; Harding, D.; Norris, G. Liquid chromatography-electrospray ionization mass spectrometry for the simultaneous quantitation of collagen and elastin crosslinks. *J. Chromatogr. A* **2016**, *1478*, 60−67.
(20) Kliment, C. R.; Englert, J. M.; Crum, L. P.; Oury, T. D. A novel method for accurate collagen and biochemical assessment of pulmonary tissue utilizing one animal. *Int. J. Clin. Exp. Pathol.* **2011**, *4* (4), 349−355.
(21) Maynes, R. *Structure and function of collagen types*; Elsevier, 2012.
(22) Miles, C. A.; Burjanadze, T. V.; Bailey, A. J. The kinetics of the thermal denaturation of collagen in unrestrained rat tail tendon determined by differential scanning calorimetry. *J. Mol. Biol.* **1995**, *245* (4), 437−446.
(23) Bozec, L.; Odlyha, M. Thermal denaturation studies of collagen by microthermal analysis and atomic force microscopy. *Biophys. J.* **2011**, *101* (1), 228−236.
(24) Flandin, F.; Buffevant, C.; Herbage, D. A differential scanning calorimetry analysis of the age-related changes in the thermal stability of rat skin collagen. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1984**, *791* (2), 205−211.
(25) Weir, C. E. Rate of shrinkage of tendon collagen-heat, entropy and free energy of activation of the shrinkage of untreated tendon. Effect of acid salt, pickle, and tannage on the activation of tendon collagen. *J. Am. Leather Chem. Assoc.* **1949**, *42*, 17−32.
(26) Bigi, A.; Cojazzi, G.; Roveri, N.; Koch, M. Differential scanning calorimetry and X-ray diffraction study of tendon collagen thermal denaturation. *Int. J. Biol. Macromol.* **1987**, *9* (6), 363−367.
(27) Wess, T. J.; Orgel, J. P. Changes in collagen structure: drying, dehydrothermal treatment and relation to long term deterioration. *Thermochim. Acta* **2000**, *365* (1−2), 119−128.
(28) Tomlin, S.; Worthington, C. In *Low-angle X-ray diffraction patterns of collagen*, Proceedings of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, The Royal Society: 1956; 189−201.

DOI: 10.1021/acssuschemeng.8b00954
ACS Sustainable Chem. Eng. 2018, 6, 7096−7104

(29) Wright, B. A. Low-angle X-ray diffraction pattern of collagen. *Nature* **1948**, *162* (4105), 23.
(30) Ascenzi, A.; Bigi, A.; Koch, M.; Ripamonti, A.; Roveri, N. A low-angle X-ray diffraction analysis of osteonic inorganic phase using synchrotron radiation. *Calcif. Tissue Int.* **1985**, *37* (6), 659−664.
(31) Bünger, M. H.; Foss, M.; Erlacher, K.; Hovgaard, M. B.; Chevallier, J.; Langdahl, B.; Bünger, C.; Birkedal, H.; Besenbacher, F.; Pedersen, J. S. Nanostructure of the neurocentral growth plate: Insight from scanning small angle X-ray scattering, atomic force microscopy and scanning electron microscopy. *Bone* **2006**, *39* (3), 530−541.
(32) Camacho, N. P.; Rinnerthaler, S.; Paschalis, E.; Mendelsohn, R.; Boskey, A.; Fratzl, P. Complementary information on bone ultrastructure from scanning small angle X-ray scattering and Fourier-transform infrared microspectroscopy. *Bone* **1999**, *25* (3), 287−293.
(33) Sykes, R. L. Modification of some reactive groups of collagen and the effect on the fixation of tervalent chromium salts. *J. Am. Leather Chem. Assoc.* **1956**, *51* (5), 235−244.
(34) Wu, B.; Mu, C.; Zhang, G.; Lin, W. Effects of Cr3+ on the structure of collagen fiber. *Langmuir* **2009**, *25* (19), 11905−11910.
(35) Bonar, L. C.; Glimcher, M. J. Thermal denaturation of mineralized and demineralized bone collagens. *J. Ultrastruct. Res.* **1970**, *32* (5−6), 545−557.
(36) Miles, C.; Bailey, A. Thermally labile domains in the collagen molecule. *Micron* **2001**, *32* (3), 325−332.
(37) Miles, C. A.; Ghelashvili, M. Polymer-in-a-box mechanism for the thermal stabilization of collagen molecules in fibers. *Biophys. J.* **1999**, *76* (6), 3243−3252.
(38) Liu, M.; Ma, J.; Lyu, B.; Gao, D.; Zhang, J. Enhancement of chromium uptake in tanning process of goat garment leather using nanocomposite. *J. Cleaner Prod.* **2016**, *133*, 487−494.
(39) Shoulders, M. D.; Raines, R. T. Collagen structure and stability. *Annu. Rev. Biochem.* **2009**, *78*, 929−958.
(40) Stats NZ. Overseas merchandise trade datasets, http://archive.stats.govt.nz/browse_for_stats/industry_sectors/imports_and_exports/overseas-merchandise-trade/HS10-by-country.aspx (accessed April 5, 2018), Harmonised System Code: 4104111511.

DOI: 10.1021/acssuschemeng.8b00954
ACS Sustainable Chem. Eng. 2018, 6, 7096−7104