Paper A2

Specific ion effects to explore the difference of chromium and aluminum salts in the stabilization of collagen

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Abstract: To explore the basis for the difference in the tanning action of the various chromium salts, such as $Cr_2(SO_4)_3$, $CrCl_3$, $Cr(NO_3)_3$, or $Cr(ClO_4)_3$, and aluminum salt, i.e. $Al_2(SO_4)_3$, we have investigated the stability of collagen in the presence of the salts based upon thermodynamic consideration. It is known that the shrinkage temperature (Ts) of the $Cr_2(SO_4)_3$, $CrCl_3$, $Cr(NO_3)_3$ and $Cr(ClO_4)_3$ tanned leather is about 115 °C, 95 °C, 75 °C, and 70 °C, respectively. This can be explained with the typical anion Hofmeister series which represents an ordering of specific ion effects, namely, $SO_4^{2-} > Cl^- > NO_3^- > ClO_4^-$. Here, the Ts is reduced gradually according to the series. In the case of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$, US-DSC studies show that the thermal stability of collagen in pH 4 solutions is reduced with the addition of $Al_2(SO_4)_3$, whereas it increases slightly in the presence of $Cr_2(SO_4)_3$. Besides, the collagen solution with a concentration of 0.5 mg/mL is salted out in 1 mM $Cr_2(SO_4)_3$ solution, but it is dissolved in $Al_2(SO_4)_3$ solution until the salt concentration is exceed 200 mM. The results indicate that Cr^{3+} has a strong salting-out effect on collagen, analogous to the kosmotropes in the Hofmeister series which is known as 'water structure makers' and exerts stabilizing and salting-out effects on proteins. AFM measurements further show that collagen fibrils aggregate after the introduction of $Cr_2(SO_4)_3$, but tend to swell in the presence of aluminum salt.

Keywords: $Cr_2(SO_4)_3$; $Al_2(SO_4)_3$; thermal stability; collagen; Hofmeister series.
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Introduction

Animal hides or skins, whose major constituent is collagen, are commercially used as natural frameworks in medical, food, and leather industries[1-4]. They are converted into leather upon tanning. Among solo tannages, the effect of $Cr_2(SO_4)_3$ exhibits unique in conferring high hydrothermal stability and desirable functional properties[5-6]. But the essential mechanism of chromium tanning is poorly understood. Presumably, the more stable coordinate bonds between bi- or polynuclear chromium ions and ionized carboxyl groups of collagen side chains is mainly responsible for the most effective tanning effect[7]. However, the process how the chemical reaction affects the tanning effect is not known. There is a perception that the weak interaction between Al and collagen is easier to break down during shrinking than the stable Cr-collagen interaction; this difference has ever been thought of as an explanation for the difference for aluminium and chromium tanning agent in the stabilization of collagen[5]. Covington et. al demonstrated that the metal tanning bonds(Al-collagen, Cr-collagen) didn't break down under shrinking condition based on energy consideration[5] and NMR tests[8]. On further studying the shrinking reaction from enthalpic interaction and entropic impact consideration, they

concluded that the chemical cross-link results in the formation of various size of cooperating unit. It is proposed that small cooperating unit leads to lower shrinking rate and higher shrinkage temperature[9]. This theory provided an effective direction to explain the various extents in improving the hydrothermal stability of raw collagens treated with different tanning agents. But the details about the formation of the cooperating unit are still poorly understood. It is accepted that the hydrothermal stability of unmodified and chemically modified collagen is dependent on the moisture content and water is crucial for the collagen helix conformation[10-12]. Covington et al further proposed that the metal ions and counterions exhibit synergistic effect in stabilizing the supramolecular water sheath around the collagen triple helix, which is called link-lock model[6,9].

Hofmeister effect is originally used to describe the interactions among ion, water and macromolecules[13]. It was first noted in 1888 that the ability of salts to precipitate certain proteins from an aqueous solution follows a recurring trend[14]. The typical anion order of Hofmerster series(HS) is: $CO_3^{2-}>SO_4^{2-}>S_2O_3^{2-}>H_2PO_4^{-}>F^{-}>Cl^{-}>Br^{-}\approx NO_3^{-}>I^{-}>ClO_4^{-}>SCN^{-}$. The species on the left of Cl^{-} are referred to as kosmotropes, which is known as 'water structure makers' and exerts stabilizing and salting-out effects on proteins. Whereas those on the right are called chaotropes, which is known as 'water structure breakers' and tend to act as protein denaturants and increase protein solubility[13-16]. Note that the shrinkage temperature (Ts) of the $Cr_2(SO_4)_3$, $CrCl_3$, $Cr(NO_3)_3$ and $Cr(ClO_4)_3$ tanned leather is about 115, 95, 75, and 70 °C, respectively, which is reduced gradually according to the series. There have been little reports about cations series because their effect is generally smaller than anions in solution[15]. However, $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ exhibits prodigious difference in conferring hydrothermal stability of collagen fiber. The cation specific effects of Cr^{3+} and Al^{3+} might be responsible for the difference stability of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ tanned leather. In addition, the stability of native collagen in the first instance can be attributed to its secondary conformation, within which hydrogen bonding[17] and inductive effects are mainly related[18]. On the other hand, the quinary structure, referred to the packing of the triple helices into fibrils, known as a 'polymer in a box ' model, providing a further level of stabilization[19].

In the present work, we used ultra sensitive differential scanning calorimetry(US-DSC) and circular dichroism (CD) to investigate the $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ induced conformational change of collagen in dilute solution based on specific ion effect consideration. We also examined the microfibrils and collagen fibers in the presence of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ by AFM. Our aim is to explore the origin of chromium tanning mechanism based on the research of collagen molecule level and hierarchical structure.

Experimental section

Materials

The acid soluble collagen used in this study was extracted from the fresh adult bovine Achilles tendon in 0.5 M acetic acid with pepsin. The details can be found in our previous work[3]. $Cr_2(SO_4)_3.6H_2O$ and $Al_2(SO_4)_3.18H_2O$ were purchased from aladdin reagent company (Shanghai, China) and used without further purification. All chemical reagents used in this work were of analytical grade. The deionized

water used during the experiment is produced with a UP Water Purification System (Ulupure, Shanghai, China) and have a minimum resistivity of 18.2 MΩ.cm.

Sample preparation

$Cr_2(SO_4)_3$, $Al_2(SO_4)_3$ and 1mg/mL collagen solution were freshly prepared with 12.4 mM sodium acetate buffer (pH 4), respectively. Equal volumes of collagen solutions and corresponding salt solutions were mixed uniformly to get homogeneous and transparent solutions. The concentration of $Al_2(SO_4)_3$ in the $Al_2(SO_4)_3$–collagen solutions ranged from 0mM to 200mM, while the concentration of $Cr_2(SO_4)_3$ in the $Cr_2(SO_4)_3$–collagen solutions varied from 0mM to 1mM. The final concentration of collagen is 0.5 mg/mL for all the samples. The solutions were kept at 4 °C for 24h before the tests.

Ultra-Sensitive Differential Scanning Calorimeter Measurements

Collagen solution with $Al_2(SO_4)_3$ or $Cr_2(SO_4)_3$ salt was measured on a VP-DSC microcalorimeter (Microcal, Northampton, USA) with the corresponding salt as the reference. Baseline controls were obtained with degassed corresponding salt solutions in both sample and reference chambers, and subtracted from sample runs. The sample solution and the reference solution were degassed for 30 min at ambient temperature (25 °C) before tests. All of the scans were conducted from 20-60 °C at 1 °C/min. The calorimetric enthalpy (ΔH_{cal}) was calculated from the area under each peak. The melting temperature (T_m) was taken as that centered at the transition[20-21]. The starting temperature (Ts) of collagen denaturation was taken as the onset of the transition.

CD tests

Collagen solutions (with a concentration of 0.2mg/mL) in the presence of $Al_2(SO_4)_3$ or $Cr_2(SO_4)_3$ salt were prepared in 12.4 mM sodium acetate buffer (pH 4). All the solutions were sealed and equilibrated in room temperature for 24h before tests. The CD spectra measurements in the far UV region from 190 to 240nm were carried on a J-810 CD spectropolarimeter (Jasco) using a quartz cell of 0.1cm light path. Each sample was scanned for 3 times at a speed of 20nm/min. The spectrum that corresponding salt solution scanned under the same condition was used as the baseline. After substracted the reference, the final spectra was expressed in terms of molar ellipticity (Em) as a function of the wavelength (λ)[2].

AFM observations

Atomic force microscopy (AFM) was used to detect the torpographic change of collagen in the presence of $Al_2(SO_4)_3$ or $Cr_2(SO_4)_3$ salt. The sample preparation for the observation of collagen microfibrils by the introduction of $Al_2(SO_4)_3$ or $Cr_2(SO_4)_3$ salt has been described detailedly in our previous report[3]. Note that the concentration of $Al_2(SO_4)_3$ or $Cr_2(SO_4)_3$ salt are both ranged from 0 to 250 μmol/g (Al^{3+}or Cr^{3+}/collagen). The preparation of pickled and tanned pigskin has been described in our previous research[2]. The frozen slices with a thickness 10nm were prepared on a cryostat microtome(CM1950, Leica-Microsystems Corporation) at -25°C. The slices were gently put onto the mica substrate and slowly air dried at 25℃ before the tests. All of the tests were proceed on a Dimension 3100 Nanoscope IV which is equipped with Silicon Tesp cantilevers (SPM-9600,

Shimadzu, Japan) in a non-contact (taping) mode[3]. For each sample, the tests were carried out at least three points to confirm the consistency of the results.

Opacity Measurements

The salt concentration required for initiation salting out collagen is characterized by the opacity of collagen solution, which is measured at 430 nm using a Specoll-11 spectrophotometer at 20 °C[22].

Results and discussion

Table 1 shows the solubility of collagen in $Al_2(SO_4)_3$ and $Cr_2(SO_4)_3$ salt. The collagen solution with a concentration of 0.5 mg/mL is salted out in 1 mM $Cr_2(SO_4)_3$ solution, but it is dissolved in $Al_2(SO_4)_3$ solution until the salt concentration is exceed 200 mM. It seems that Cr^{3+} has a strong salting-out effect on collagen, analogous to the kosmotropes in the Hofmeister series(HS), which is known as 'water structure makers' and exerts salting-out effect on protein. However, the Al^{3+} has a salting-in effect on collagen, similar to the chaotropes in the HS, which is known as 'water structure breakers' and tend to act as protein denaturants and increase protein solubility[13-16].

Table 1 The minimum concentration of various salts required to precipitate 0.5 mg/mL collagen from 12.4 **mM sodium acetate buffer(pH 4)**

Salt	Collagen salting-out concentration(mM/L)
$Al_2(SO_4)_3$	200
$Cr_2(SO_4)_3$	1

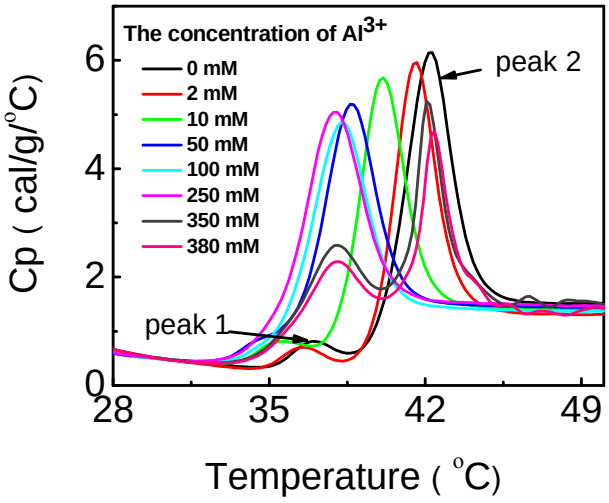

Fig. 1. Temperature dependence of the heat capacity (Cp) of collagen in 12.4 mM sodium acetate buffer (pH 4) with various concentration of Al^{3+} (a: 0 mM, b: 2 mM, c: 10 mM, d: 50 mM, e: 100 mM, f: 250 mM, g: 380 mM)

The dependence of thermal stability of collagen in 12.4 mM sodium acetate buffer (pH 4) on the concentration of $Al_2(SO_4)_3$ has been studied by US-DSC (Fig. 1). As reported before, two endothermic peaks with a pre-transition at ~36 °C and a main denaturational transition at ~42 °C is observed for collagen in acidic solution[22-23]. With the addition of Al^{3+}, three concentration ranges can be clearly distinguished in the dependence of collagen thermal stability on the ion concentration, which is similar to the effect of chaotropic[22] on the stability of macromolecule.

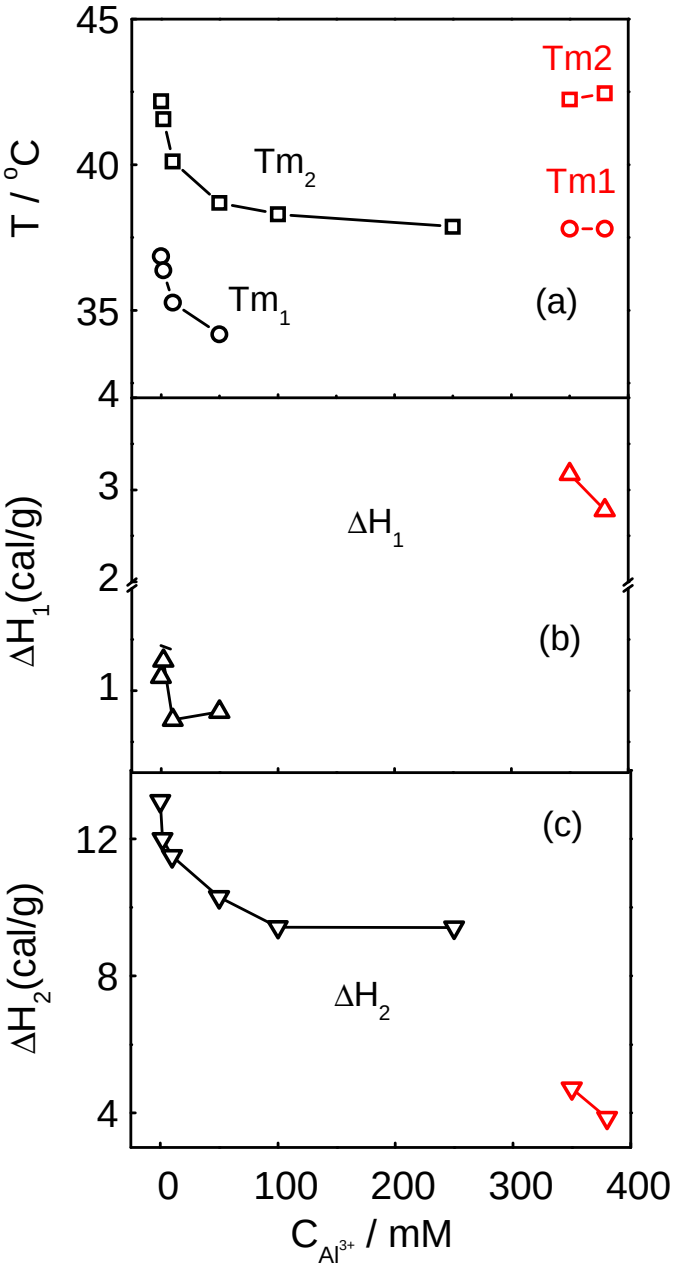

Fig. 2. The concentration of Al^{3+} dependence of the transition temperature (Tm1, Tm2) and the enthalpy change (ΔH1, ΔH2)

As the concentration of Al^{3+} is below 50 mM, both the pre-transiton and major transition shift to lower temperatures in parallel, which is resulting from the screening of the electrostatic interactions between the charged amino acid residues of collagen (Fig. 2a). On further increase of the Al^{3+} concentration from 50 mM to 250 mM, the temperature of collagen unfolding (Tm_2) keep on reducing, which is due to the Hofmeister effects on the stability of macromolecules. The Hofmeister effect originates from the competition between the macromolecule surface groups and the solutes[24]. By destabilizing the structure of bulk water, the chaotropic solutes tend to increase the interface area and destabilize protein. However, the kosmotropic solutes tend to act in the opposite way to stabilize the structure of bulk water[22]. At further higher Al^{3+} concentration, a sharp increase of the denaturation temperature is observed, which is accompanied by the increase of the sample opacity (data not show). The partial salting-out and aggregation of collagen might be responsible for the simultaneous increase in denaturation temperature (Tm_1, Tm_2) and the sample opacity. It is interesting to note that the main transition splits into two peaks at Al^{3+} concentration 100 mM and 250 mM. Such splitting was also observed in previous report[22] for sulphate salts. It could be due to the specifically interactions between the sulphate anions with the collagen molecules.

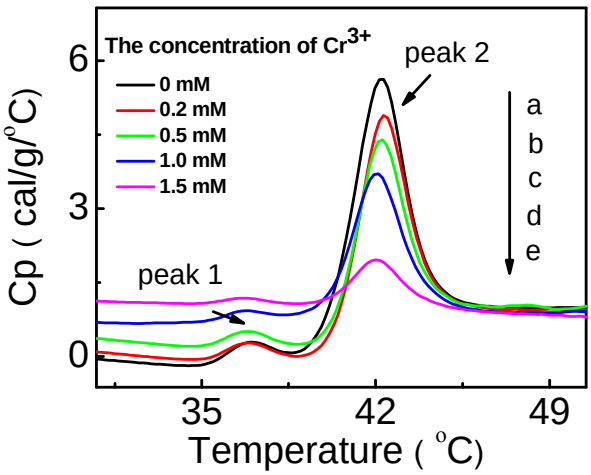

Fig. 3. Temperature dependence of the heat capacity (Cp) of collagen in 12.4 mM sodium acetate buffer (pH 4) with various concentrations of Cr^{3+} (a: 0 mM, b: 0.2 mM, c: 0.5 mM, d: 1.0 mM, e: 1.5 mM)

The effect of $Cr_2(SO_4)_3$ concentration on the thermal stability of collagen has been also examined (Fig. 3). With the addition of Cr^{3+}, the positions of pre-transition and main transition (Tm_1, Tm_2) almost don't change. Whereas the starting temperature of main transition (Tp_2) increased slightly (Fig. 4a), indicating the thermal stability of collagen is improved by the introduction of $Cr_2(SO_4)_3$. Fig. 4 shows the enthalpy change for both peaks (ΔH_1, ΔH_2) decreases with the addition of $Cr_2(SO_4)_3$, suggesting that partial hydrogen bonds of collagen helix are broken. It is notable that the heat capacity for native collagen (Cp_1) increased as the Cr^{3+} concentration, whereas the heat capacity corresponding to the unfolded state (Cp_2) remains the same value. It is known that the Cp represents the hydrophobicity of macromolecules, a high Cp means hydrophobicity, whereas a low value indicates

hydrophilicity[25-29]. Note that the hydrogen bond concerning water is crucial for stabilizing the collagen triple helix structure at the molecule level of native collagen structure. Here the substitution of water for Cr^{3+} might be responsible for the increased hydrophobicity.

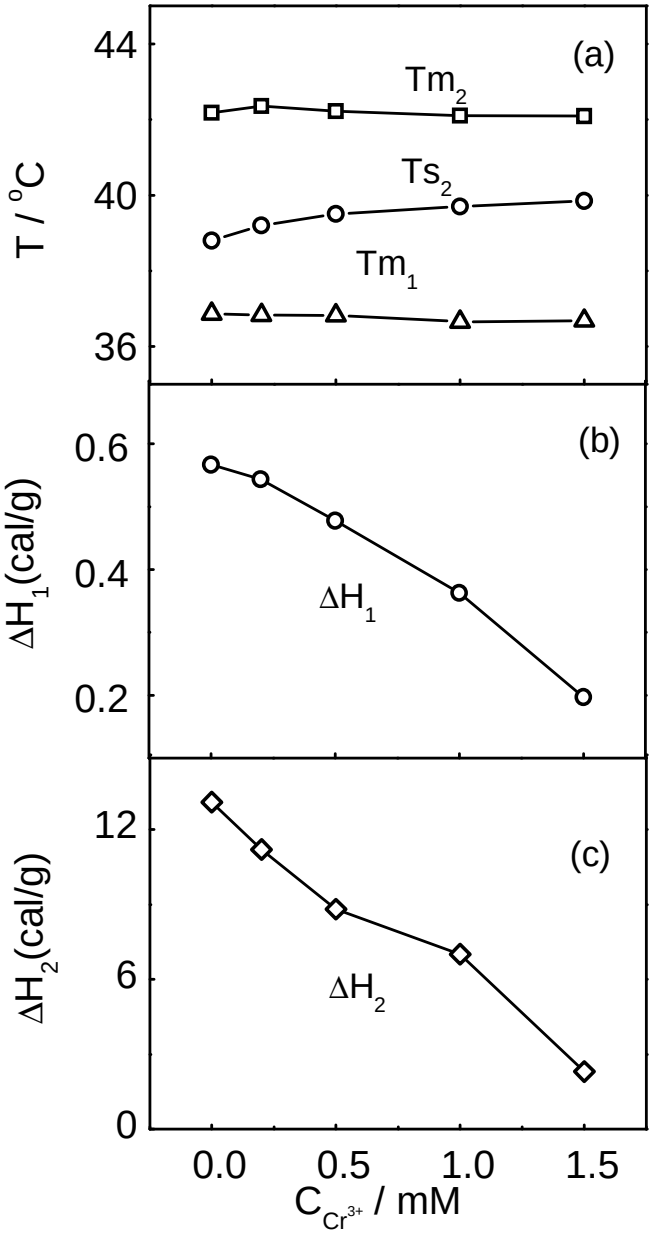

Fig. 4. The concentration of Cr^{3+} dependence of the transition temperature (Tm1, Tm2) and the enthalpy change (ΔH1, ΔH2)

In order to investigate whether the $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ induce any alterations in the conformation of collagen, CD spectral studies on collagen solution in the presence of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ have been carried out. Normally, in the far UV region, the complete unfolding of collagen results in the completely disappearance of the positive peak at 220nm and the negative band at 198nm

red shift[2,30]. Partially denatured collagen leads to red shift of crossover points and a low Rpn (the ratio of positive peak intensity to the negative peak intensity). The increase of Rpn indicates that the collagen molecule is folded or aggregated in solution[30-32]. With the addition of Al^{3+}, the position and the intensity of the positive peak slightly change, the Rpn values increase a little. This is likely due to the Al^{3+}-collagen aggregates, which is consistent with the US-DSC results. However, when $[Cr^{3+}]$ < 0.2 mM, the position and intensity of the positive peak almost don't change, so does the Rpn (Fig. 5, Tab. 2), indicating that the introduction of Cr^{3+} doesn't destroy the triple helix structure of collagen. This is consistent with the previous report[2,31]. Further increase in $[Cr^{3+}]$ resulted in a slight red shift of crossover point and a little decrease of Rpn value, suggesting that Cr^{3+} induced partially denaturation of collagen. It is known that $Cr_2(SO_4)_3$ is the most effective tanning agent in converting hide or skin to leather. The Cr^{3+} ion can form multiple covalent cross-links with collagen by reacting with the ionized sidechain carboxyl groups. Here it is conceivable that the reaction of Cr^{3+} with the COO^- groups along one peptide chain or between chains within a triple helix can leads to the structural distortion of the collagen helix[33]. The postulation has already been demonstrated by the WXAD tests for collagen fibers treated with Cr^{3+} salts[2].

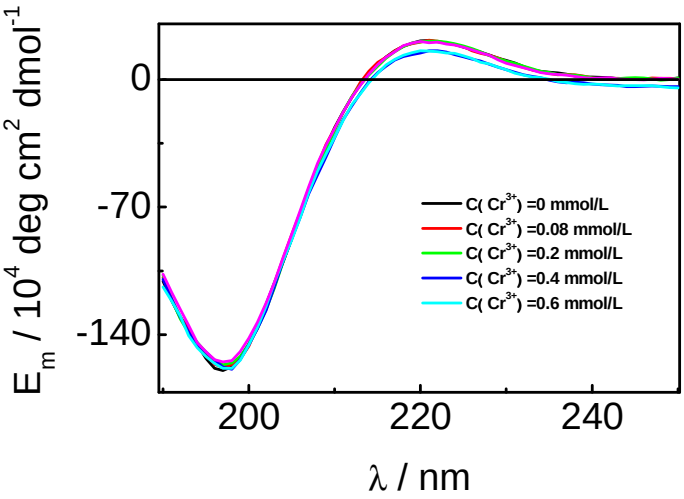

Fig. 5 Far-UV spectra of 0.2mg/mL collagen solution in the presence of Cr^{3+} ion

Tab. 2 CD data for collagen solutions in 1 mM acetic buffer (pH 4.01) in the presence of Cr^{3+}

$[Cr^{3+}]$/(mM)	$\theta_{\lambda, max}$/nm	max (Em)/10^4 deg cm^{-2} $dmol^{-1}$	$\theta_{\lambda, min}$/nm	max (-Em)/10^4 deg cm^{-2} $dmol^{-1}$	Rpn	Crossover point
0	220	21.56	197	159.62	0.135	213.42
0.08	220	21.59	197	157.82	0.137	213.25
0.2	220	21.56	197	156.63	0.138	213.58
0.4	222	16.65	198	158.42	0.105	214.22
0.6	221	16.09	197	158.43	0.101	214.34

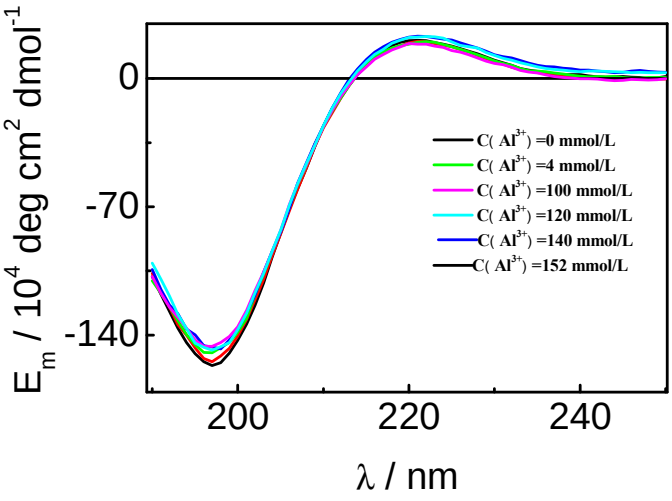

Fig. 6 Far-UV spectra of 0.2mg/mL collagen solution in the presence of Al^{3+} ion

Tab. 3 CD data for aqueous collagen solutions in 1mM acetic buffer (pH 4.01) in the presence of Al^{3+}

$[Al^{3+}]$/(mM)	$\theta_{\lambda, max}$/nm	max (Em)/10^4 deg cm^{-2} $dmol^{-1}$	$\theta_{\lambda, min}$/nm	max (-Em)/10^4 deg cm^{-2} $dmol^{-1}$	Rpn	Crossover point
0	220	21.56	197	159.62	0.135	213.42
4	221	20.36	197	154.49	0.132	213.62
100	221	19.98	197	149.84	0.133	213.67
120	221	23.20	198	147.85	0.157	213.10
140	221	22.39	197	148.28	0.151	213.31
152	221	19.85	197	145.67	0.136	213.65

AFM tests have been carried out to examine the morphological changes of collagen induced by the addition of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$. Typical fibrillar structure with many curved molecules or microfibrils lie and are overlapped with one another are observed on the mica substrate for the pure type I collagen (Fig. 7a and 8a), which is similar to previous reports[3,34]. While in the presence of Cr^{3+}, the collagen microfibril appears as mesh-like fibrous network topographies, suggesting the occurrence of aggregation. As the Cr^{3+} increased from 25umol/g to 250umol/g, the width of the microfibrils increase from ~50nm to ~200nm (Fig. 7b, 7c and 7d). It seems that the collagen molecules tend to aggregation by the introduction of Cr^{3+}, which is induced by dehydration because of the covalent cross-link between Cr^{3+} and ionized sidechain carboxyl groups of collagen. However, with the addition of Al^{3+}, the microfibrils appear to intertwine with each other in a sheet-like mesh tend to swell, which might be due to the water structure breaking effect of chaopic.

The effect of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ on the morphologies of collagenous matrix have also been examined by AFM. The pure collagen fiber exhibits a macroscopically disordered network structure (Fig. 9a). And the alternative dark (~0.4D overlap) and light (~0.6D overlap) banding pattern can be

observed clearly at a higher magnification (Fig. 9b). Here the D periodicity is ~60 nm, which is consistent with the previous reported value (~60nm observed with AFM)[32,35-36]. Fig. 10 shows that the introduction of $Al_2(SO_4)_3$ didn't alter the D periodicity. Whereas it decreased to ~50 nm with the addition of $Cr_2(SO_4)_3$ (Fig. 11), which is agreement with the reported value (reduced ~9 nm treated with chromium dimer)[32]. It is acceptable that Cr^{3+} ion can form more stable multiple covalent cross-link with collagen by reacting with the ionized sidechain carboxyl groups. And the moisture content has a great influence on the thermal stability of collagen fiber. It is conceivable that the displacement and dehydrate of water involved in hydration network of collagen by chromium might be responsible for the observed difference in D-periodicty.

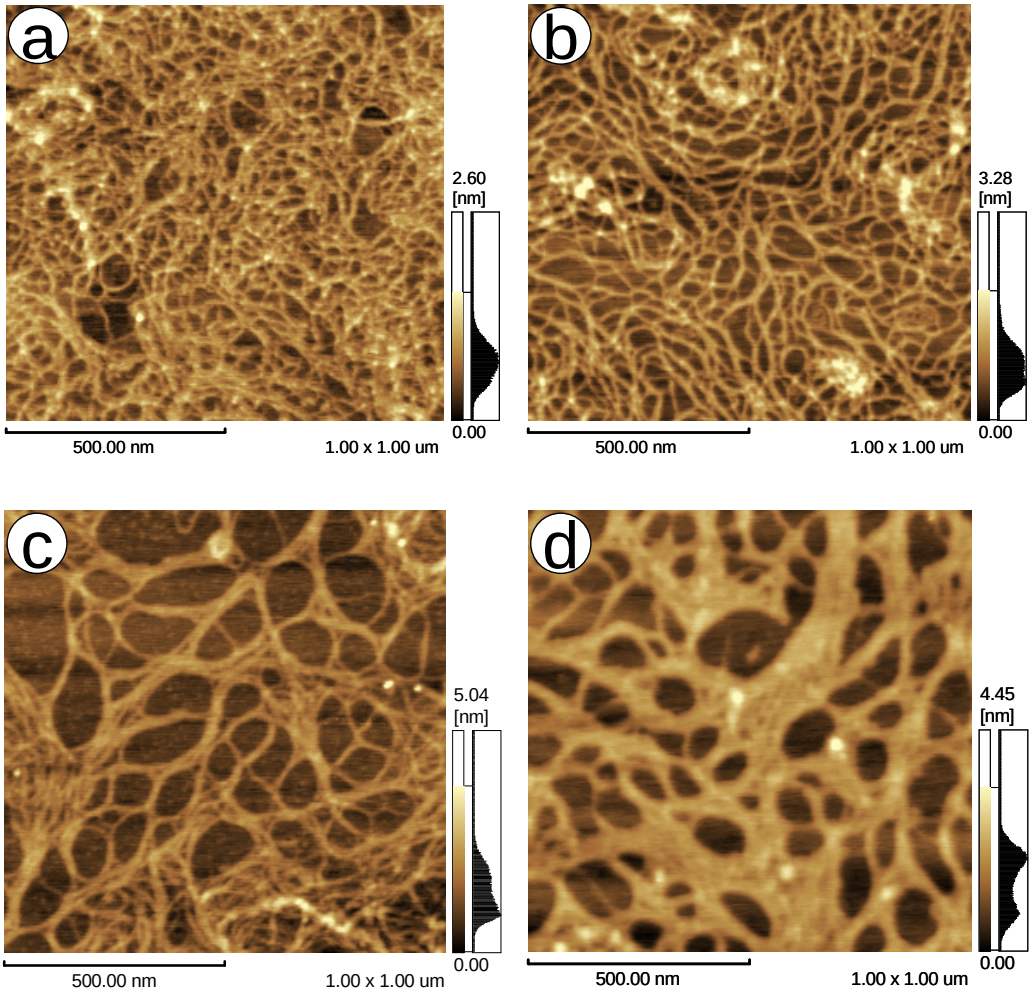

Fig. 7 AFM images of collagen treated with different concentrations of $Cr_2(SO_4)_3$: (a) 0; (b) 25 µmol/g; (c) 125 µmol/g;(d) 250 µmol/g (Cr^{3+}/collagen).

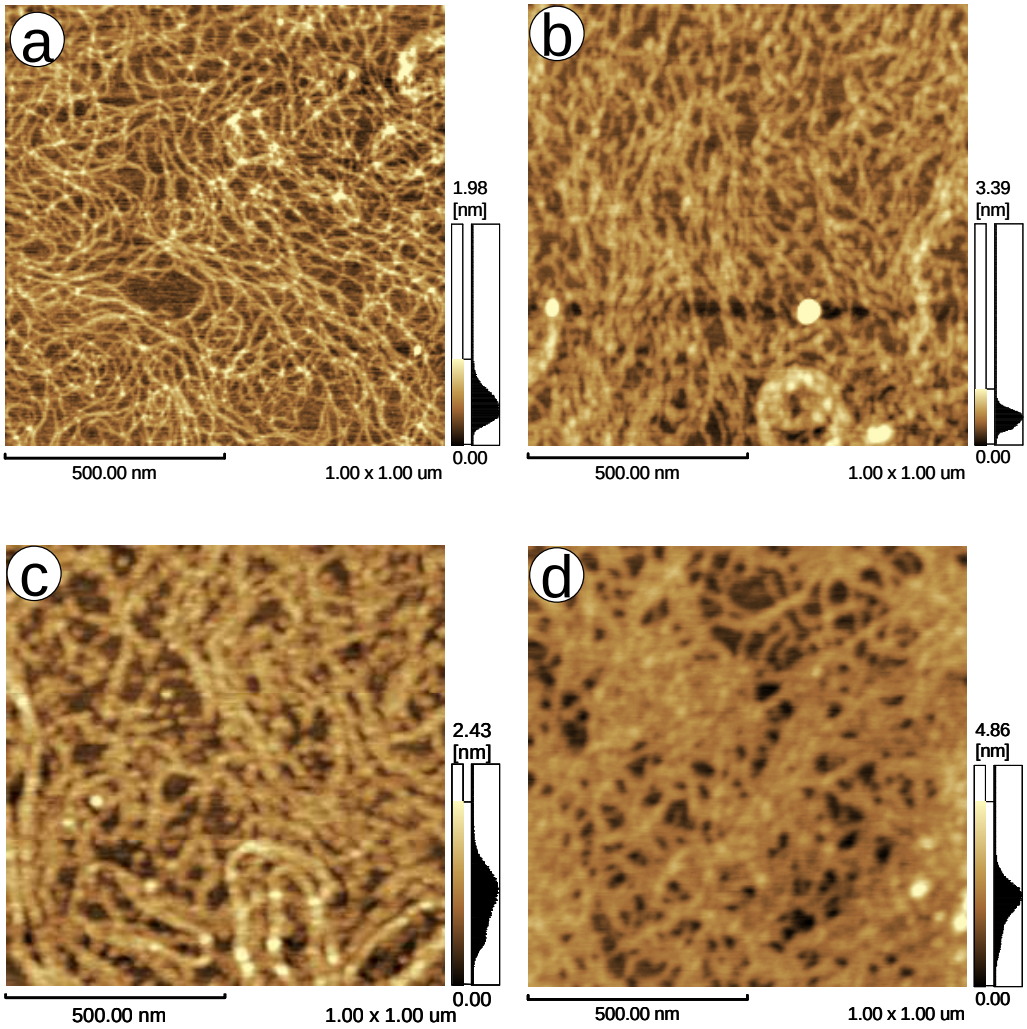

Fig. 8 AFM images of collagen treated with different concentrations of $Al_2(SO_4)_3$: (a) 0; (b) 25 µmol/g; (c) 125 µmol/g;(d) 250 µmol/g (Al^{3+}/collagen).

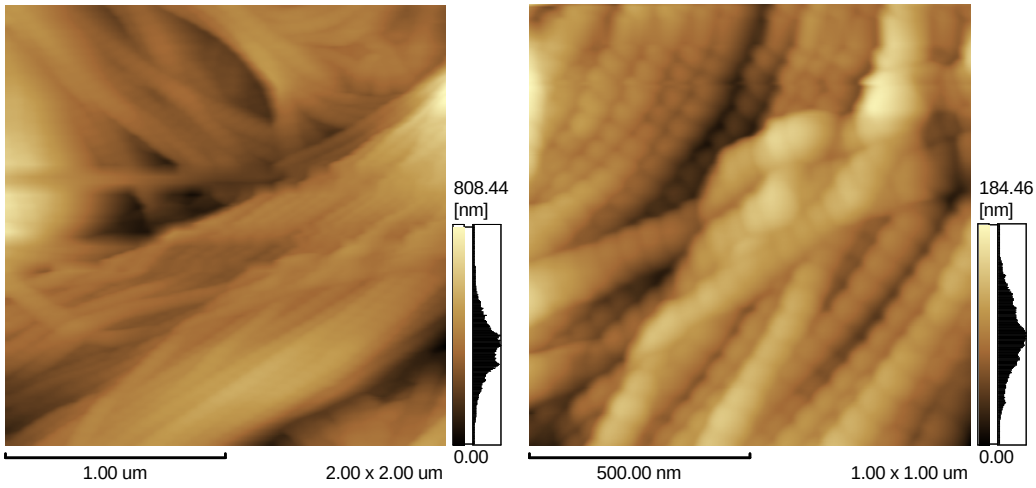

Fig. 9 The AFM images of pickled goatskin

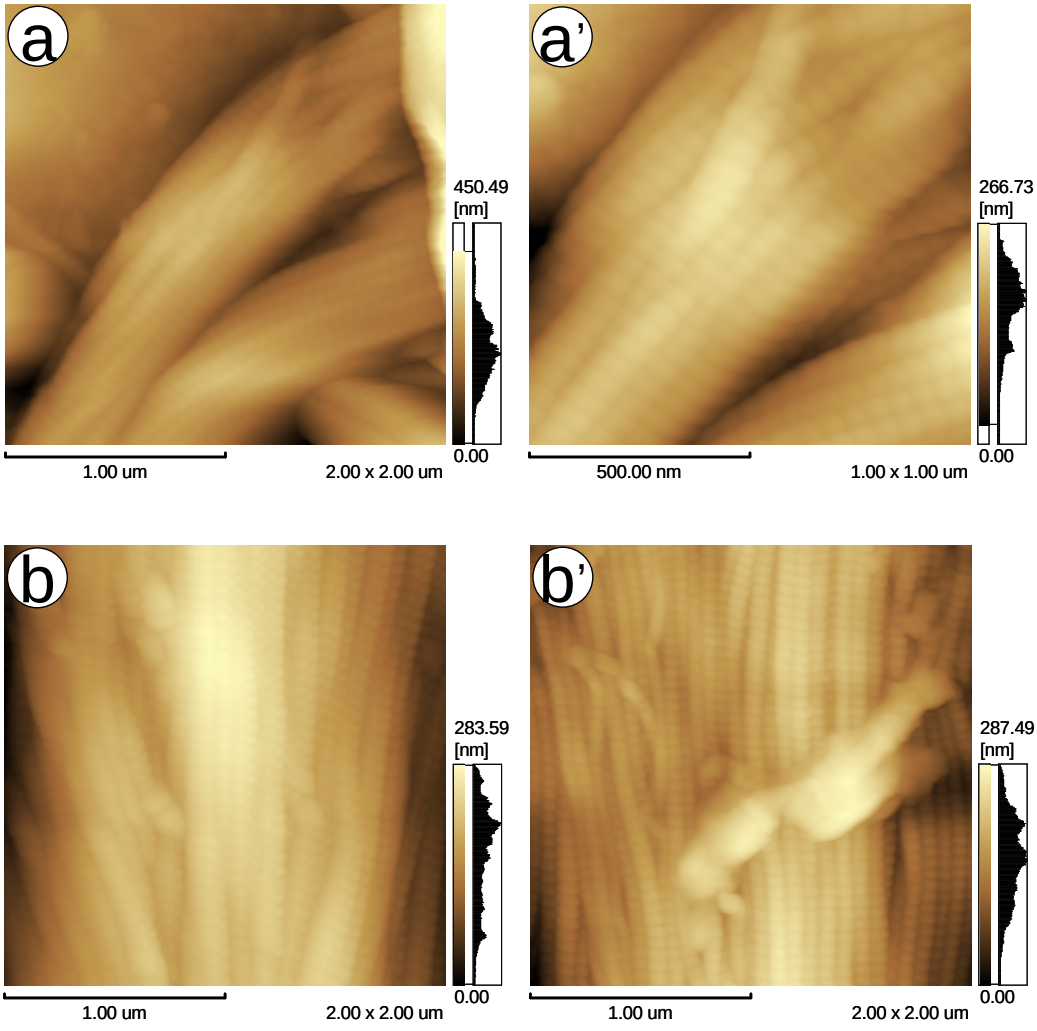

Fig. 10 AFM images of leather tanned with $Al_2(SO_4)_3$

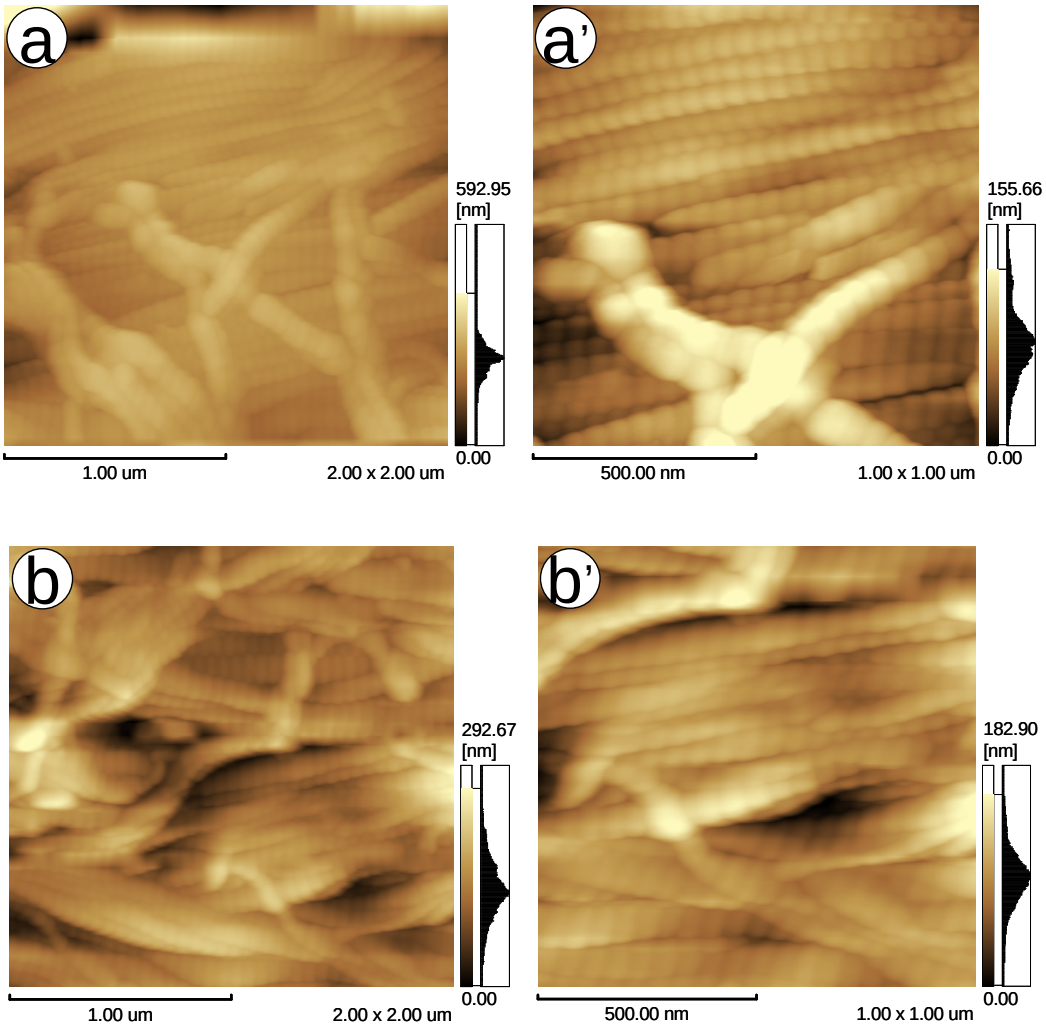

Fig. 11 AFM images of leather tanned with $Cr_2(SO_4)_3$

Conclusions

The studies on the collagen molecule and collagen hierarchical structure in the presence of $Cr_2(SO_4)_3$ and $Al_2(SO_4)_3$ can lead to the following conclusions. The different effect of Cr^{3+} and Al^{3+} on water is mainly responsible for the difference in the stabilization of collagen. On the one hand, Cr^{3+} can displace the water concerning the stabilizing collagen triple helix. On the other hand, Cr^{3+} ion can form more stable multiple covalent cross-link with collagen by reacting with the ionized sidechain carboxyl groups, leading to the dehydration between collagen fibers. $Cr_2(SO_4)_3$ has a strong salting-out effect on collagen, analogous to the kosmotropes in the Hofmeister series which is known as 'water structure makers'. US-DSC shows that $Cr_2(SO_4)_3$ can slightly enhance the thermal stability of collagen in solution by displacing the water corresponding to the stabilizing the collagen triple helix structure. CD spectra reveals that the introduction of $Cr_2(SO_4)_3$ leads to the slightly distortion of collagen molecule but didn't disrupt the helix conformation. AFM measurements for collagen microfibrils indicate that collagen fibrils aggregate after the introduction of $Cr_2(SO_4)_3$, but tend to swell in the presence of the

$Al_2(SO_4)_3$. Collagen fiber observations by AFM show that D periodicity reduced from ~60 nm to ~50 nm with the addition of $Cr_2(SO_4)_3$, but remains ~60 nm after the introduction of $Al_2(SO_4)_3$.

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