Study on Interaction Mechanism between Neutral Salts and Collagen by Combining Experiments with Molecular Dynamics Simulation

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Abstract

The effect of salt on the collagen of hide/skin is of great significance in leather-making. However, the interaction between neutral salts and collagen has not been clear, since the microscopic interaction is hard to be observed directly from the macro level of hide/skin collagen. In this study, the collagen solutions in the typical neutral salts (NaCl, $CaCl_2$, and Na_2SO_4) systems were used to explore the interaction mechanism between neutral salts and collagen via combining experiments with molecular dynamics (MD) simulation. The results of fluorescence measurements of pyrene, dynamic light scattering, atomic force microscopy, and isoelectric point suggested that the variation of the interaction between different neutral salts and collagen was accompanied with the changes in physicochemical properties of collagen. MD simulation further revealed more detailed information on the interaction mechanism between neutral salts and collagen at the molecular level. The computational results of non-bond energy of the collagen-salt model boxes indicated that the electrostatic interactions of different salts with collagen molecules had the order of $CaCl_2$> Na_2SO_4> NaCl. The analyses of the visualized conformation and the radial distribution functions showed that $CaCl_2$ with Ca^{2+} as contributing ion tended to form intramolecular salt bridges with collagen, while Na_2SO_4 with SO_4^{2-} as contributing ion more likely formed salt bridges between collagen molecules in the shape of agglomerates. In contrast, NaCl with Cl^- as contributing ion was scattered around the collagen models, and its effect on collagen was much smaller. The study elaborated the interaction mechanism of typical neutral salts and collagen to be helpful for further understanding and improving the use of neutral salts in many steps involved in leather production.

Introduction

Neutral salts, as common chemicals used in leather production, play an important role in the production of leather with satisfactory mechanical, aesthetic and hygienic performance.[1] The effects of neutral salts on pelts in leather-making processes have been investigated, which were known as the salt effects such as avoiding undesirable swelling, dispersing fiber bundles, dehydrating, and reducing the thickness variance of pelts.[1-3] Although the effects of neutral salts in leather-making processes have been known, the underlying interaction mechanism between neutral salts and collagen in leather is not fully understood. Figuring out the interaction mechanism between neutral salts and collagen would be helpful to understand the roles of salts and provide guidance to make full use of salts in leather-making industry.

Collagen is the main structural component within leather and hide/skin in the form of high-level fiber bundles.[4-6] Since the microscopic interaction is hard to be directly observed from the macro level of hide/skin collagen fibers, collagen solution is a better option to explore the interaction mechanisms. The physicochemical properties of collagen, such as microstructure, thermal stability, isoelectric point, viscosity, fiber-forming properties, and so on, would be changed with the addition of neutral salts.[7-11] Wei et al. studied the microstructure of collagen molecules in NaCl solution to describe the role of NaCl in pickling and tanning processes.[12] The formation of an orderly and porous microstructure was observed via atomic force microscope (AFM) when NaCl was present in collagen solution, which was considered to help improve the penetration of tanning agents and the mechanical property of leathers.[12] Brown et al. investigated the effect of neutral salts on the hydrothermal stability of collagen, and the results suggested that the neutral salts appeared to bind to collagen via electrostatic interaction to decrease the hydrothermal stability of collagen.[13] Penkova et al. further detected the thermal stability of collagen varied with salt ion species as followed the order of $H_2PO_4^-$≥ SO_4^{2-}> Cl^-> SCN^-, indicating salts had different interactions with collagen.[14] Freudenberg et al. found that $CaCl_2$ shifted the isoelectric point of collagen to a higher pH, indicating calcium ions bound to the carboxyl groups of collagen molecules.[15] Duan et al. deduced that the decline of collagen viscosity at low concentrations of NaCl solutions was attributed to the change of interaction among collagen molecules due to the charge screening by chloride ions.[9] Li et al. proposed that the main interaction between multivalent ions and collagen was ion binding and the multivalent ions could change collagen surface charge after investigating the fiber-forming properties of collagen in various salts.[10] These studies have established an association between salts and the physicochemical

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properties of collagen solutions, which suggested that neutral salts had electrostatic interactions with collagen molecules and the interactions between salts and collagen varied with salt species. However, the interaction mechanism between collagen and neutral salts is not completely clear, and there is little information about the interaction mechanism at molecular level.

Molecular dynamics (MD) simulation is an effective method to explore interaction mechanism of particles at molecular level.[16, 17] This technology can simulate various experimental conditions, such as changes in solvent, to understand the conformation changes and dynamic mechanisms of proteins.[18] At present, MD simulation has been increasingly applied in research on collagen to give insight into the microscopic interaction mechanisms of collagen under various conditions.[19, 20] Niu et al. explored the mechanism of collagen intrafibrillar mineralization via using MD simulation to analyze the movement data of water and ions in the collagen mineralization models in presence of polyelectrolyte ions.[20] Ding et al. performed MD simulation on a chrome tanning model containing collagen fibrils and polychromiums, and clearly described the cross-linking site, topology and capacity of the cross-linkage of polychromiums to peptide chains.[21] Bulo et al. built a collagen fibril model and simulated the pickling process to study the effects of neutral salts (NaCl, $CaCl_2$, and Na_2SO_4) on the swelling behavior of fibrils in pickling.[4] Through the RDF analysis, it confirmed that $CaCl_2$ and Na_2SO_4 had specific stabilizing effects on fibrils owing to the weak self-interaction of $CaCl_2$ and the strong interactions of SO_4^{2-} with the basic amino acid residues of fibrils, respectively.[4] These studies mainly used collagen fibril models for investigation, some detailed information at the molecular and atomic levels could not be obtained. And there are few studies using a collagen molecule model to investigate the interaction mechanism between salts and collagen. A MD simulation with a collagen molecule model would be helpful to explore the mechanism between neutral salts and collagen with a deep understanding.

In this study, the interaction mechanism between typical neutral salts (NaCl, $CaCl_2$, and Na_2SO_4) and collagen was investigated by combining experiments with MD simulation. Pyrene fluorescence spectra, dynamic light scattering (DLS) measurements, and AFM were applied to characterize the effect of the neutral salts on the intermolecular interactions between collagen molecules in different salt solutions based on the aggregation behaviors of collagen molecules. Zeta potential measurement was performed to detect the change of isoelectric point (pI). MD simulation with a collagen molecule model was performed to get some insight into the interaction mechanisms between neutral salts and collagen at molecular level, including energy calculation, conformation analysis and RDF calculation. The results were expected to further understand and improve the use of neutral salts in many steps involved in leather production.

Materials and Methods

Materials

Collagen was extracted from bovine hide following the method of Li et al. with a slight modification.[22] Briefly, after unhairing and defatting pretreatments, the dermis of bovine hide was cut into small pieces, and the pieces were dissolved in 0.5 mol/L acetic acid containing 3% pepsin (1:3000) at 4°C. Type I collagen was collected and purified from the supernatants by centrifuging, salting out, redissolving, dialyzing and lyophilizing. The collagen sponge was stored in a desiccator for use.

Pepsin and pyrene were purchased from Sigma Aldrich.

Preparation of Collagen Solutions with Salts

Lyophilized collagen was dissolved in 0.01 M acetic acid to obtain 1.0 mg/mL and 3.0 mg/mL collagen stock solutions. Then the 1.0 mg/mL collagen stock solutions were separately mixed with NaCl solutions at different concentrations at 4°C to finally obtain 0.5 mg/mL collagen sample solutions containing a series of NaCl concentrations (0, 40, 80, 120, 160, and 200 mM). The collagen sample solutions containing 0–200 mM $CaCl_2$ or Na_2SO_4 were similarly prepared by replacing NaCl with $CaCl_2$ or Na_2SO_4. The collagen sample solution containing 0 mM salt was used as the control, simply called pure Col, and the collagen sample solutions containing NaCl, $CaCl_2$ or Na_2SO_4 were called Col/NaCl, Col/$CaCl_2$, and Col/Na_2SO_4, respectively. These sample solutions were subjected for the subsequent measurements. And the 3.0 mg/mL collagen stock solutions were separately mixed with salt solutions to obtain 0.001–3.0 mg/mL collagen sample solutions containing 80 mM NaCl, $CaCl_2$, or Na_2SO_4, which were used for collagen critical aggregation concentration (CAC) measurements.

Measurements of Pyrene Fluorescence Spectra

Pyrene fluorescence spectra measurements were performed to characterize collagen aggregation state and determine the CAC of collagen. Fluorescence spectra measurements were performed according to the method of Yang et al with slight modifications.[23] Firstly, 50 μL 400 μM pyrene solution was introduced to a 25-mL brown glass volumetric flask and gently evaporated under a nitrogen gas stream. Then the collagen sample solutions (pure Col, Col/NaCl, Col/$CaCl_2$, and Col/Na_2SO_4) were separately added into the volumetric flasks and fully homogenized by an ultrasonic cleaner at 4°C. Before measurement, the solutions were kept in a dark place for 12 h at 4°C. Measurements were performed with a fluorescence spectrophotometer (F-7100, Cary Eclipse, Agilent, Santa Clara, California) at 25°C. All samples were excited at 343 nm, and the emission spectra were collected from 360 to 460 nm with a scanning rate of 240 nm/min. In the measurements, the slits opening of excitation and emission were fixed at 5 nm and 2.5 nm, respectively. All fluorescence measurements were performed in triplicate.

Measurements of Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were carried out for the size distribution of the collagen solutions. After being transferred to polystyrene cuvettes, the 0.5 mg/mL collagen solutions containing 0–200 mM salts were immediately determined by a Zetasizer instrument (Nano-ZS, Malvern, UK) with an angle detection of 90° at 25°C. All DLS measurements were carried out in triplicate.

Atomic Force Microscope Measurements

The 0.5 mg/mL collagen solutions containing 0–200 mM salts were diluted to 25 μg/mL for AFM measurements. Droplets of the diluted solutions were immediately dropped on freshly cleaved mica sheets and then dried at room temperature for 48 h in air. The microstructure of the dried samples was observed by AFM (Shimadzu SPM 9600, Kyoto, Japan) in the soft tapping mode with a scanning rate of 1HZ.

Measurements of Zeta Potential

The 0.5 mg/mL collagen solutions containing 0 mM and 80 mM salts were determined for zeta potential. Firstly, the pH of these solutions was adjusted to 4~11 with 0.2 M NaOH under ice-bath conditions. Then, these solutions were immediately measured for the zeta potential by a Zetasizer instrument (Nano-ZS, Malvern, UK) at 25°C. The Smoluchowski model was chosen to calculate the zeta potential. Each sample was determined in triplicate.

Constructions of Collagen Model and Simulation Boxes

Collagen molecule model and simulation box constructions, and molecular dynamics (MD) simulation were performed by the Materials Studio, version 6.0 software package (Key Laboratory of the Ministry of Education for Advanced Catalysis Materials, Institute of Physical Chemistry, Zhejiang Normal University, Zhejiang, China).

The amino acid sequence of bovine hide type I collagen was retrieved from the UniProtKB database (entry: P02453(α1), P02465(α2)). Due to the big length of the collagen chains, we selected a fragment of the collagen chains as natural collagen molecule model based on the content of ionizable amino acids, and its amino acid sequence was listed:

α_1→GEQGVPGDLGAPGPSGARGERGFPGERGVQ

α_2→GERGIPGEFGLPGPAGARGERGPPGESGAA

α_1→GEQGVPGDLGAPGPSGARGERGFPGERGVQ

The collagen molecule model was constructed according to the method of the previous work in our lab with a slight modification.[24] The basic amino acid residues of the model were protonated and the acidic amino acid residues were dissociated accompanied with 12 free H^+ in order to accelerate the interactions between salts and the collagen model. The model was further refined by geometry optimization with a smart algorithm in Forcite module. H_2O, CH_3COO^-, H^+ and salt ions (Na^+, Cl^-, Ca^{2+}, and SO_4^{2-}) were also built and geometry optimized.

The final simulation system for the control group (simply called [pure Col] system) consisted of a collagen model, 3 CH_3COO^-, 15 H^+, and 500 H_2O, and they were randomly placed in a cubic box with a density of 1 g/cm^3 by Amorphous cell module. NaCl, $CaCl_2$, and Na_2SO_4 were added and randomly dispersed into the [pure Col] system to constitute the corresponding systems, simply called [Col/NaCl], [Col/Na_2SO_4] and [Col/$CaCl_2$] systems, respectively, and the number of salts added to each system was 20. The simulation boxes were geometry optimized by Forcite module.

Molecular Dynamics Simulation

To investigate the interaction mechanism between the salts and collagen, the MD simulations with an NPT ensemble (298.15 K and 1 atm) were carried out by Forcite module. The Dreiding force field was applied in the simulation, and the Berendsen barostat and the Nose-Hoover thermostat were used to keep the pressure and temperature constant, respectively. The MD simulations consisted of two simulations, the first one for an equilibration run and the latter one with 50 000 steps for a production run. All analyses were calculated based on the production run. The non-bond energy of collagen models in different salt systems was calculated and the conformations of collagen models in each system were used to analyze the interactions between collagen models and salt ions. The final 500 ps trajectories of the production run were for radial distribution function (RDF) analysis which could describe the distribution of the salt ions and water molecules around the collagen model. The RDFs of the collagen model with salt ions and water were obtained by choosing corresponding particles as the sets to calculate, and the cutoff distance between the sets for RDFs was 30 Å.

Results and Discussion

Pyrene Fluorescence Spectra Analysis

As a sensitive hydrophobic fluorescent probe, pyrene can be used to measure the polarity of the microenvironment of solutions.[25] Since collagen aggregation was accompanied by the formation of hydrophobic microdomains where pyrene was located, pyrene fluorescence spectra can be employed to characterize collagen aggregation behaviors and intermolecular interactions in solutions.[23]

Figure 1A1–A3 shows the pyrene fluorescence emission spectra of 0.5 mg/mL collagen solutions containing 0–200 mM salts (NaCl, $CaCl_2$, and Na_2SO_4). The spectra display the four characteristic peaks at 374,

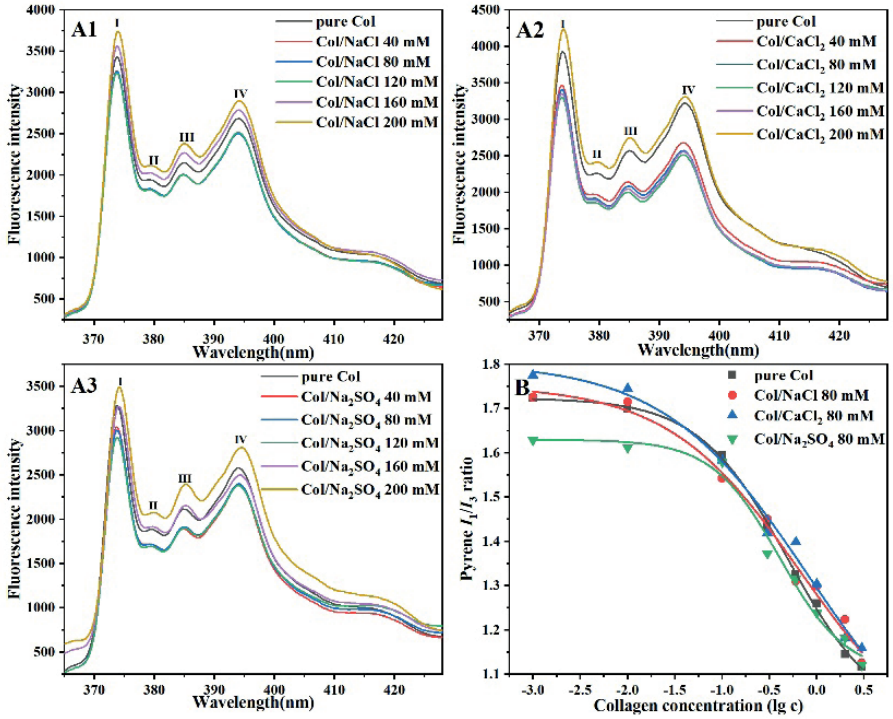

Figure 1. Pyrene fluorescence emission spectra in 0.5 mg/mL collagen solutions containing 0–200 mM salts (**A1**–**A3**). **A1**, Col/NaCl; **A2**, Col/$CaCl_2$; **A3**, Col/Na_2SO_4. Plots of the pyrene I_1/I_3 ratio versus the logarithm of the collagen concentration in 0 mM and 80 mM salts (NaCl, $CaCl_2$, and Na_2SO_4) solutions at 25°C (**B**).

380, 385 and 394 nm, indicating pyrene was stable in solutions.[25] The intensity ratio of the first to the third vibronic peaks (I_1/I_3) could be regarded as a polarity index.[25] The I_1/I_3 values of the collagen solutions containing 0–200 mM salts are listed in Table I. From Table I, we found that in all studied salt solutions, with increasing salt concentration, the I_1/I_3 values first increased, suggesting the decrease of the hydrophobic microdomains, which was correlated with the disaggregation of collagen molecules and the weak intermolecular interactions. However, with further increasing salt concentration, the I_1/I_3 values turned to decrease, indicating the enhancement of collagen aggregation and intermolecular interactions. Besides salt concentration, the I_1/I_3 values and aggregation behaviors were also affected by salt species. It was noted that the fewest hydrophobic microdomains and weakest aggregation behavior of collagen molecules were at the salt concentrations of 40 mM for Na_2SO_4, 120 mM for NaCl and 160 mM for $CaCl_2$, respectively. Compared with the I_1/I_3 values in Col/NaCl, Col/$CaCl_2$, and Col/Na_2SO_4 at the same salt concentration, it was speculated that the collagen aggregation degree and interactions with salts in salt solutions had the order of Na_2SO_4> NaCl> $CaCl_2$. Furthermore, the order was consistent with the ability of these salts to suppress acid swelling in leather-making, which may be explained by that the salts at high concentrations enhanced collagen intermolecular interactions, promoted collagen aggregation, made water between collagen molecules out and then led to suppress pelts swelling. NaCl had a relative moderate ability to promote aggregation, so it was often used to suppress swelling, while Na_2SO_4 was used to dehydrate pelts and $CaCl_2$ was mainly used to disperse fibers of leather.[1] From the results, it was concluded that collagen aggregation behavior was affected by the concentration and species of salts and the salts affected the electrostatic and hydrophobic interactions of collagen molecules since aggregation was the result of these interactions.

Table I

The I_1/I_3 Ratio Values of Pyrene in 0.5 mg/mL Collagen Solutions Containing 0–200 mM Salts (NaCl, $CaCl_2$ and Na_2SO_4) and the CAC Values of Collagen in 0 mM and 80 mM Salts

Salt concentration (mM)	pure Col	Col/NaCl	Col/$CaCl_2$	Col/Na_2SO_4
0	1.599±0.005	1.599±0.005	1.599±0.005	1.599±0.005
40	/	1.613±0.001	1.621±0.004	1.605±0.003
80	/	1.617±0.002	1.632±0.004	1.569±0.004
120	/	1.621±0.001	1.644±0.001	1.538±0.004
160	/	1.571±0.004	1.648±0.005	1.525±0.003
200	/	1.497±0.007	1.542±0.005	1.456±0.004
CAC (mg/mL)	0.502	0.667	0.816	0.402

In order to further characterize the different effects caused by salt species, the CAC values of collagen in 0 mM and 80 mM salts were determined by pyrene fluorescence spectra method. The fluorescence spectra of 0.001–3.0 mg/mL collagen solutions with 80 mM salts also showed the characteristic peaks of pyrene (data not shown). The plots of the I_1/I_3 values versus the logarithm of the collagen concentration in different salts are presented in Figure 1B. By curve fitting the plots with a Boltzmann function, the concentration where the I_1/I_3 value was 50% of the initial I_1/I_3 value could be regarded as the CAC of collagen.[23] The CAC values of collagen in 0 mM and 80 mM salts are summarized in Table I. The CAC of pure Col was 0.502 mg/mL, consistent with the result of Yan et al.[25] The CAC values of collagen with 80 mM NaCl, $CaCl_2$, and Na_2SO_4 were 0.667, 0.816, and 0.402 mg/mL, respectively, indicating at 80 mM salt concentration, collagen molecules were induced to aggregate by Na_2SO_4, disperse slightly by NaCl and strongly disperse by $CaCl_2$. And it further indicated that at 80 mM, collagen intermolecular interactions were enhanced by Na_2SO_4, weakened slightly by NaCl and strongly weakened by $CaCl_2$, which implied collagen molecules had different interaction mechanisms with different salts.

Dynamic Light Scattering (DLS) Analysis

DLS can characterize the size distribution of collagen molecules and aggregates in salt solutions (NaCl, $CaCl_2$, and Na_2SO_4). Figure 2 displays the size distribution of 0.5 mg/mL collagen solutions containing 0–200 mM salts. All collagen solutions show two distribution ranges with values of 10–150 nm (called region A) and 460–2670 nm (called region B), which might be ascribed to the collagen aggregates with various sizes and the rod-like shape of collagen molecule.[26] Changes in the range and intensity of the two regions were observed in these sample solutions. For Col/NaCl (Figure 2B1–B5), the intensity of region B decreased and region B shifted to a smaller size region when NaCl concentration increased from 0 mM to 120 mM. Whereas, when NaCl further increased to 200 mM, the changes in size distribution were opposite to the above changes. It indicated that NaCl induced the disaggregation of collagen aggregates at 40–120 mM, but at higher concentrations (160–200 mM) NaCl promoted aggregation due to the enhanced collagen interactions. In Figure 2C1–C4 (Col/$CaCl_2$), region B obviously shifted to a smaller size region with increasing $CaCl_2$ concentration, indicating collagen was dispersed by 40–160 mM $CaCl_2$, which was ascribed to the increase of electrostatic repulsions between collagen molecules. Figure 2D2–D5 (Col/Na_2SO_4) shows the regions A and B both shifted to regions with larger particle sizes as Na_2SO_4 concentration increased, suggesting that Na_2SO_4 enhanced collagen interactions and promoted aggregation. The intensity of region B slightly reduced, which might be deduced that the formation of larger aggregates was accompanied with the disaggregation of some aggregates. The DLS results agreed with the results of pyrene fluorescence spectra. From the aspect of the aggregates size distribution, DLS results directly confirmed that collagen aggregation behavior and interactions could be modulated by these salts.

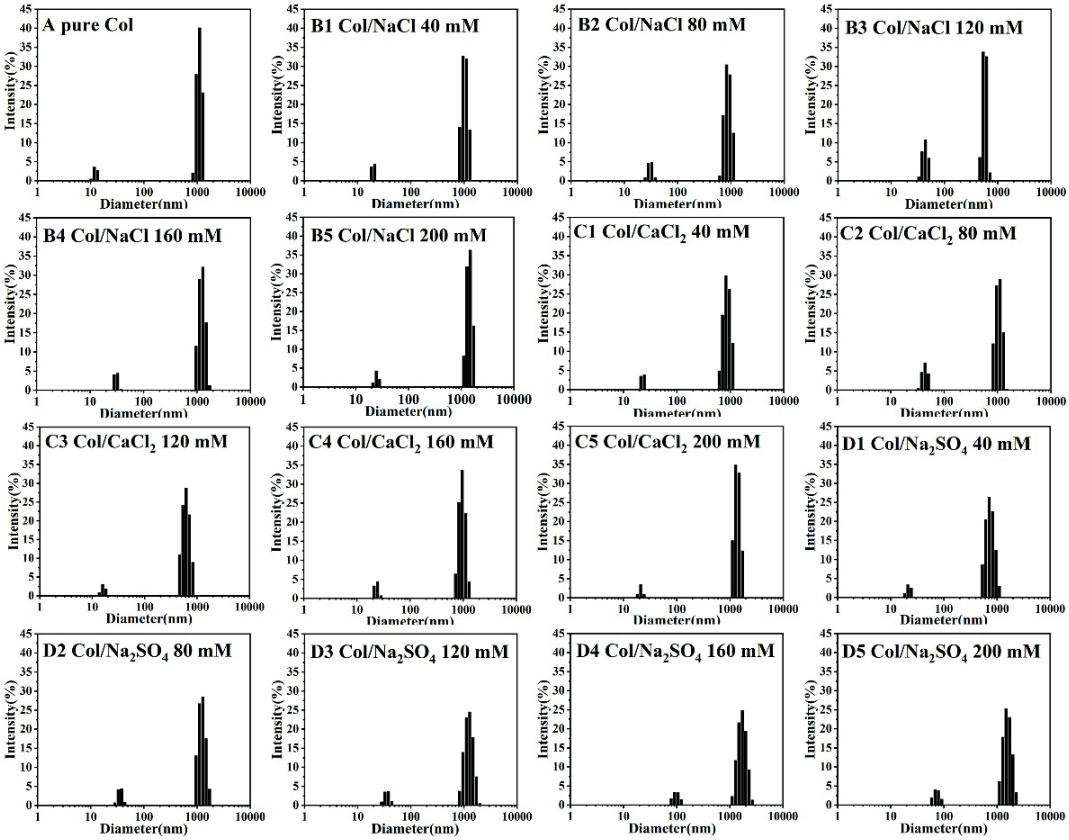

Figure 2. The size distribution of 0.5 mg/mL collagen solutions containing 0–200 mM salts by DLS at 25. **A**, pure Col; **B1–B5**, Col/NaCl; **C1–C5**, Col/$CaCl_2$; **D1–D5**, Col/Na_2SO_4.

Atomic Force Microscope (AFM) Images

Figure 3 displays the AFM images and morphological changes of collagen in 0–200 mM salt solutions. The collagen concentration (0.5 mg/mL) was close to collagen CAC (0.502 mg/mL), so it could be observed that some collagen molecules entangled with each other and aggregated in Figure 3A. Figure 3B1–B3 shows a disordered and loose morphology of collagen at 40–120 mM NaCl, while with more NaCl, collagen molecules were induced to entangle tightly and form orderly aggregates with spaces in Figure 3B4–B5. Wei et al. reported a similar phenomenon and ascribed it to the dehydration effect of NaCl.[12] Compared with NaCl, $CaCl_2$ induced collagen molecules to form dispersed loose porous networks in Figure 3C1–C4, and the porous structure was obvious in Figure 3C5, suggesting $CaCl_2$ had a dispersing effect on collagen. While Col/Na_2SO_4 presented tight entanglements with larger diameter of aggregates as presented in Figure 3D2–D5, especially obvious in Figure 3D5, indicating collagen interactions and aggregation were enhanced by Na_2SO_4. It was interesting to find that the morphology of collagen in different salts observed by AFM corresponded to the different effects of salts in leather-making. The AFM results were kept in line with the results of the fluorescence and DLS, and it suggested that NaCl, $CaCl_2$ and Na_2SO_4 had different effects on the micromorphology of collagen aggregates and interactions.

Zeta Potential Analysis

Zeta potential measurements were conducted to determine isoelectric point (pI) of collagen and further study the electrostatic interactions of collagen molecules in presence of salts. Figure 4 presents the zeta potential and pI of collagen in 0 mM and 80 mM salt solutions. The pI of pure Col was 7.5, similar to the result of Freudenberg et al.,[15] and the pI values of Col/NaCl, Col/$CaCl_2$, and Col/Na_2SO_4 at 80 mM salt concentration were 6.4, 10.8 and 5.5, respectively. The pI and surface charge of collagen were greatly affected by salt species, and the effect of $CaCl_2$ and Na_2SO_4 on pI was similar to the result of Li.[10] The change of pI was correlated with the preferential adsorption and binding of salt ions on collagen molecules.[10, 15, 27] The decreases of pI by NaCl and Na_2SO_4 implied that collagen mainly interacted with Cl^- of NaCl and SO_4^{2-} of Na_2SO_4. The preferential adsorption and binding of Cl^- and SO_4^{2-} on the positively charged residues of collagen, such as amino groups, reduced the positive charge of collagen, which led to the decrease of pI. At the same time the binding of the negative salt ions (Cl^- and SO_4^{2-}) to collagen could weaken the electrostatic repulsion between collagen molecules and promote collagen aggregation. On the contrary, the increase of pI by $CaCl_2$ implied that collagen had a preferential binding with positive Ca^{2+} of $CaCl_2$. The preferential binding of Ca^{2+} on the negatively charged residues of collagen, such as carboxyl groups, reduced the

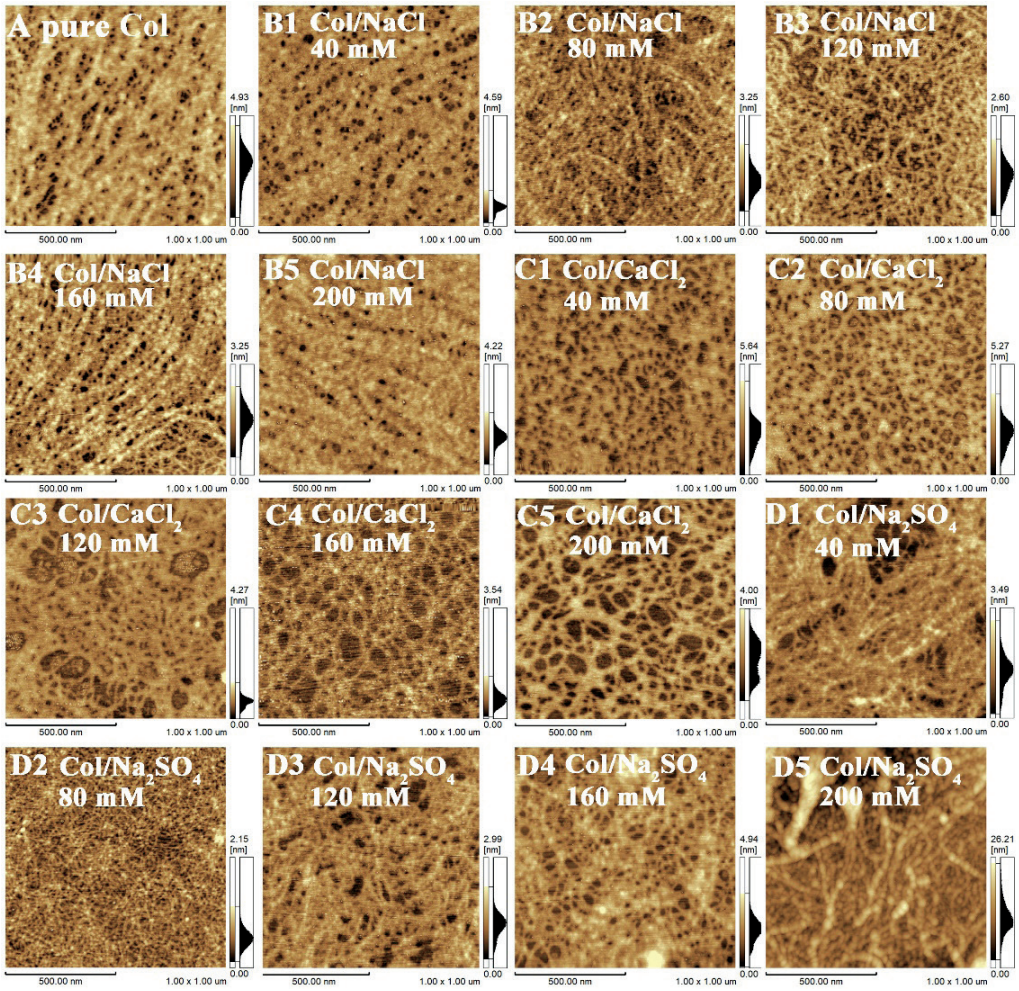

Figure 3. AFM height images of 0.5 mg/mL collagen solutions with 0–200 mM salts. **A**, pure Col; **B1–B5**, Col/NaCl; **C1–C5**, Col/$CaCl_2$; **D1–D5**, Col/Na_2SO_4.

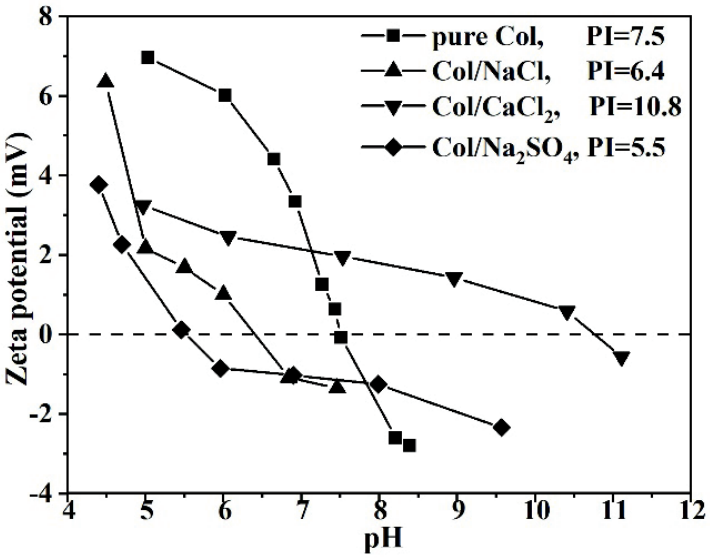

Figure 4. Zeta potential measurement of collagen in 0 mM and 80 mM salt solutions at 25°C.

negative charge of collagen, which led to the increase of pI. The binding of Ca^{2+} could enhance the electrostatic repulsion and inhibit aggregation. Compared with the monovalent salt ions (Na^+ and Cl^-), the divalent salt ions (Ca^{2+} and SO_4^{2-}) had stronger interactions with collagen because the change of pI in Col/$CaCl_2$ and Col/Na_2SO_4 was bigger than that in Col/NaCl.

Non-bond Energy Analysis in Different Salt Systems

The interactions between salts and collagen are non-bond interactions which include hydrogen bond, van der Waals and electrostatic interaction, so we calculated non-bond energies of collagen models in different salt systems to study the interactions between salts and collagen. The non-bond energies of collagen models in different salt systems are listed in Table II. As shown in Table II, the total non-bond energy of collagen models in salt systems was lower than that in [pure Col] system, and the non-bond energies including hydrogen bond, van der Waals and electrostatic energies of collagen models were affected by the salts. Also, it was observed that the change in electrostatic energy was bigger than the change in other non-bond energies, indicating the salts interacted with collagen mainly via electrostatic interactions. The magnitude of the effects of salts on electrostatic energy was ranked as NaCl< Na_2SO_4< $CaCl_2$, which was consistent with the magnitude of changes in the pI result.

Interactions between Collagen and Salts by Conformation Analysis

Molecular dynamics (MD) simulation is an effective tool to explore the interactions of collagen molecules with salt ions at molecular level and the conformation analysis from MD simulation can make the interactions visualized.[16, 17] The conformations of the different salt systems at the end of the simulation run are shown in Figure 5. The water molecules are not shown for a better visual effect, and there are two collagen models in each conformation because two boxes are displayed.

Figure 5 shows that in all salt systems, salt ions were around collagen models and had electrostatic interactions with collagen model. It was found that the salt ions bridged the acidic amino acid residues and basic amino acid residues of collagen molecules by intramolecular and intermolecular salt bridges, labeled in purple and red circles, respectively. In [Col/NaCl] system (Figure 5A), salt ions were scattered around collagen model, and there was an intermolecular salt bridge formed between the collagen models. By magnifying the intermolecular salt bridge in the red rectangle, we observed that Cl^- mainly interacted with the amino groups of collagen models to form salt bridges. The binding of Cl^- to collagen and the salt bridges could promote collagen interactions and aggregation. Figure 5B shows that in [Col/$CaCl_2$] system, Ca^{2+} and Cl^- wrapped around the collagen models with a line shape, and salt ions tended to form intramolecular salt bridges which was not beneficial for the interactions between collagen models. An intermolecular salt bridge was formed by $CaCl_2$ and Ca^{2+} mainly interacted with the carboxy groups of collagen models. The binding of positive Ca^{2+} to collagen enhanced the electrostatic repulsions between collagen models and then promoted collagen dispersion. In [Col/Na_2SO_4] system (Figure 5C), the salt ions formed clusters between the collagen models, and SO_4^{2-} mainly interacted with collagen models. Compared with NaCl and $CaCl_2$, Na_2SO_4 tended to form more intermolecular salt bridges between collagen molecules in the shape of agglomerates, indicating that Na_2SO_4 had a stronger ability to form intermolecular salt bridges and promote collagen aggregation. And the negative SO_4^{2-} directly interacted with the amino groups of collagen models, which could screen the positive charge of collagen and reduce electrostatic repulsion, as same as the results reported by Mertz et al.[28] Therefore, Na_2SO_4 could enhance collagen interactions and

Table II

Non-bond Energies (kcal/mol) of Collagen Model in [pure col] System and Different Salt Systems

System	Total non-bond energy	Hydrogen bond	van der Waals	Electrostatic energy
[pure Col]	-5657.619	-1591.960	789.922	-4855.581
[Col/NaCl]	-9439.522	-1553.262	917.032	-8803.292
[Col/$CaCl_2$]	-16944.006	-1622.528	1507.496	-16828.974
[Col/Na_2SO_4]	-15750.372	-1691.213	1035.209	-15094.369

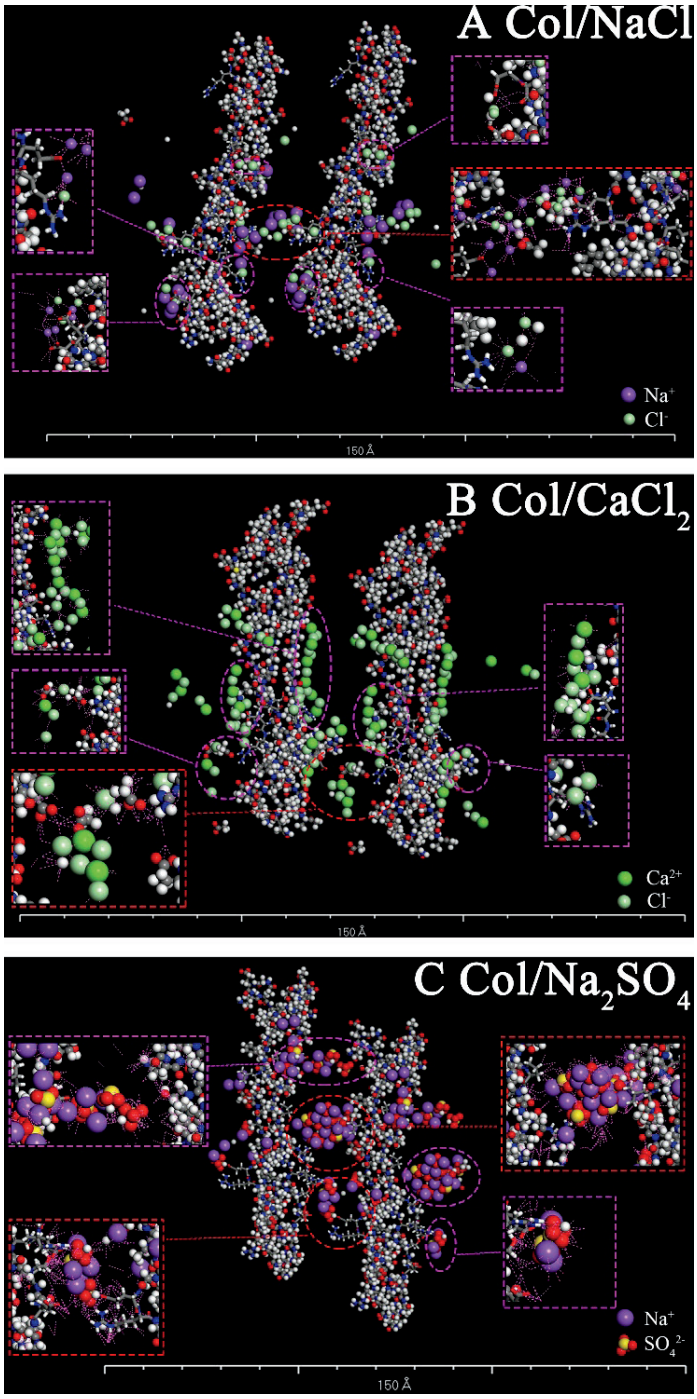

Figure 5. Conformations of collagen model in various salt systems. **A**, [Col/NaCl] system; **B**, [Col/$CaCl_2$] system and **C**, [Col/Na_2SO_4] system. Red and purple circles represented intermolecular and intramolecular salt bridges, respectively. Pink dashed lines represented electrostatic interactions.

promote aggregation due to enough intermolecular salt bridges formed in the shape of agglomerates and the screening charge effect of SO_4^{2-}.

Radial Distribution Function Analysis

Radial distribution function (RDF) is often used to analyze interactions of particles in simulation because it can describe the distribution and densities of the selected particles appearing around the collagen model.[29] The RDF values are positively correlated with the densities of the particles and they are normalized by the average densities.[4] We calculated RDFs to study the interactions between salts and collagen and the effect of salts on the hydration shell of collagen molecules.

The RDFs of Collagen–salt ions describe the distributions of salt ions around the charged amino acid residues (Glu, Asp and Arg) of the collagen model and they are shown in Figure 6A1–C2. All of the Collagen–salt ions RDFs had a peak within a distance of 5 Å and the peak values were much larger than 1, suggesting these salt ions were around the ionizable amino acid residues of collagen and had strong electrostatic interactions with the amino acid residues. In [Col/NaCl] system, the density of Cl^- around collagen was higher than that of Na^+ around collagen by comparing the peak values in Figure 6A1 and A2, indicating Cl^- of NaCl was the contributing ion for the interaction with collagen. The interaction of Cl^- with collagen was also reported by Bersusan et al.[30] Similarly, it could be inferred from Figure 6B1–C2 that Ca^{2+} of $CaCl_2$ and SO_4^{2-} of Na_2SO_4 were the contributing ions, and there were some studies reported the strong interaction of SO_4^{2-} with the charged moieties of amino acids.[28, 31] The density of Na^+ around collagen in [Col/NaCl] system (Figure 6A1) was higher than that in [Col/Na_2SO_4] system (Figure 6C1), which could be deduced that SO_4^{2-} had stronger interactions with collagen than Cl^-. Similarly, the noticeable decreased density of Cl^- in the [Col/$CaCl_2$] system (Figure 6B2) suggested Ca^{2+} had stronger interactions with collagen than Na^+. It seemed that divalent salt ions had greater effects on collagen than monovalent salt ions, which was supported by the electrostatic energy result. The results of contributing ions based on RDFs were consistent with the pI results. Since the contributing ions of NaCl and Na_2SO_4 were anions, the electrostatic repulsions between collagen molecules could be weakened by the screening charge effect of salt anions, and Na_2SO_4 had a stronger ability to promote collagen interactions than NaCl. $CaCl_2$ could enhance collagen electrostatic repulsions by the binding of positive Ca^{2+} to collagen and then disperse collagen.

The RDFs of Collagen–H_2O describe the distribution of water molecules around the collagen model in different systems and they are shown in Figure 6D–G. In [pure Col] system (Figure 6D), the first peak with a value bigger than 1 was at 3.19 Å, indicating water molecules were likely to appear around collagen molecules at this distance and it corresponded to the position of the first hydration

shell of collagen molecules. In [Col/NaCl] system (Figure 6E), the first hydration shell of collagen molecules shifted to a closer distance from collagen, at 2.93 Å, and the first peak value was larger than that in the [pure Col] system. It suggested that NaCl increased the density of water in the first hydration shell of collagen, which was the result of the weakened interactions between collagen models by NaCl. A similar change of the first peak was found in Figure 6F, and the change was more noticeable. Figure 6F suggested that $CaCl_2$ made the first hydration shell of collagen shift to 2.03 Å and observably enhanced the density of water around collagen. $CaCl_2$ had a bigger effect on the enhancement of the hydration shell of collagen than NaCl, indicating collagen molecules were more dispersed in $CaCl_2$ solution than in NaCl solution. The strong solvation effect of Ca^{2+} was also reported by Bulo et al.[4] Contrary to NaCl and $CaCl_2$, as depicted in Figure 6.G, Na_2SO_4 reduced the density of water in the first hydration shell of collagen and then excluded the water to a farther distance from collagen, at 11.79 Å, which corresponded to the position of the second hydration shell. It may be attributed to that Na_2SO_4 enhanced collagen intermolecular interactions, which made water move outward. The results of Collagen–H_2O RDF suggested that the density of water in the first hydration shell of collagen was increased a little by NaCl and clearly increased by $CaCl_2$, while clearly decreased by Na_2SO_4, which was correlated with different interactions of the salts with collagen.

The interaction mechanism between typical neutral salts (NaCl, $CaCl_2$, and Na_2SO_4) and collagen was investigated, which was helpful for understanding the different roles of the salts in leather-making. The results revealed that Ca^{2+} of $CaCl_2$, SO_4^{2-} of Na_2SO_4, and Cl^- of NaCl were the contributing ions for the interactions of the salts with collagen. $CaCl_2$ tended to form intramolecular salt bridges with collagen and the binding of Ca^{2+} enhanced the electrostatic repulsions among collagen molecules, which could widen the distance between collagen molecules and induce collagen to form loose porous networks. At the same time, more water was attracted to appear around collagen in the presence of $CaCl_2$. Then, as a result, collagen fibers and fiber bundles could be dispersed and opened up by $CaCl_2$. Gao et al. also reported that Ca^{2+} could widen the distance between fibers and disperse fibers, but Ca^{2+} also could make fibers swell and expand.[32] Na_2SO_4 obviously promoted collagen aggregation owing to clustered intermolecular salt bridges formed between collagen molecules and the weakened electrostatic repulsions by binding of SO_4^{2-}. Na_2SO_4 induced collagen to aggregate tightly, which could make water between collagen move outward, so Na_2SO_4 could dehydrate collagen fibers and fiber bundles. Owing to the strong ability of Na_2SO_4 to dehydrate and promote aggregation, fibers and fiber bundles might be not fully dispersed. Zhang et al. found that the opening effect of SO_4^{2-} on fiber bundles was not satisfying.[33] Compared with $CaCl_2$ and Na_2SO_4, NaCl had a moderate ability

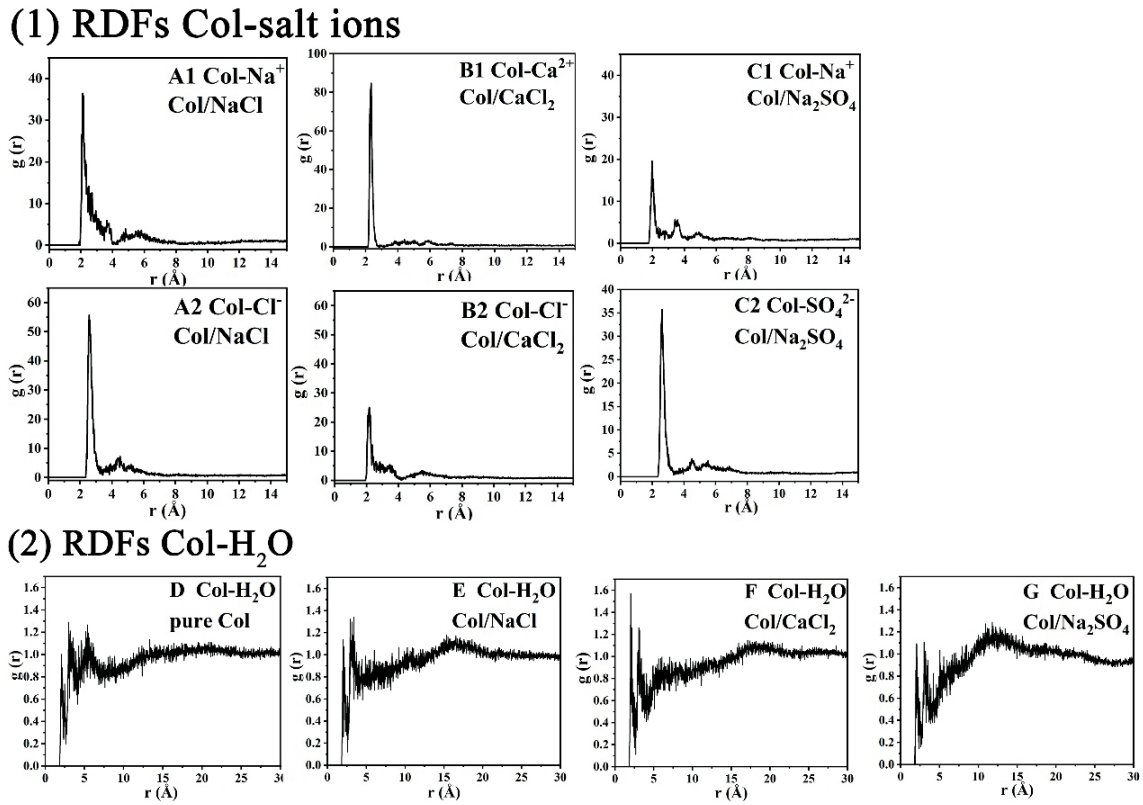

Figure 6. RDFs of collagen model with salt ions (**A1**–**C2**) and water (**D**–**G**) in different systems. **A1**, **A2**, **E**, [Col/NaCl] system; **B1**, **B2**, **F**, [Col/$CaCl_2$] system; **C1**, **C2**, **G**, [Col/Na_2SO_4] system; **D**, [pure Col] system.

to promote collagen aggregation and could induce collagen to form orderly aggregates with spaces, which could make water move outward. So when NaCl with high concentration was used in pickling, fibers would not be swollen and fiber bundles would be dispersed, which was beneficial for producing leather with good mechanical and hygienic performance.[2,5] Fibers and fiber bundles could be fully dispersed by $CaCl_2$, while fully dehydrated by Na_2SO_4, and moderately dispersed and dehydrated by NaCl.

Conclusion

The interaction mechanism between typical neutral salts (NaCl, $CaCl_2$, and Na_2SO_4) and collagen was investigated by combining experiments and MD simulation. The experimental results (Pyrene fluorescence spectra, DLS, AFM, and pI) indicated that the variation of the interaction between different neutral salts and collagen was accompanied with the changes in physicochemical properties of collagen. MD simulation further revealed that the electrostatic interactions of different salts with collagen molecules had the order of $CaCl_2$>Na_2SO_4>NaCl. Ca^{2+} of $CaCl_2$, SO_4^{2-} of Na_2SO_4, and Cl^- of NaCl were the contributing ions for the interactions of the salts with collagen. $CaCl_2$ tended to form intramolecular salt bridges with collagen and the binding of Ca^{2+} enhanced the electrostatic repulsions among collagen molecules, which led to the dispersing effect of $CaCl_2$. Na_2SO_4 tended to form intermolecular salt bridges between collagen molecules in the shape of agglomerates and the binding of SO_4^{2-} weakened the electrostatic repulsions, which promoted collagen interactions. NaCl was scattered around the collagen models, and its effect on collagen was much smaller. MD simulation with a natural collagen molecule model shed light on the mechanism between neutral salts and collagen at the molecular level. The results would provide guidance for further understanding and improving the use of neutral salts in the leather production.

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