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FULL PAPER

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Anion-regulated binding selectivity of Cr(III) in collagen

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Abstract

We present a mechanism for the selectivity of covalent/electrostatic binding of the Cr(III) ion to collagen, mediated by the kosmotropicity of the anions. Although a change in the long-range ordered structure of collagen is observed after covalent binding (Cr(III)-OOC) in the presence of SO_4^{2-} at pH 4.5, the $\nu_{sym}(COO^-)$ band remains intense, suggesting a relatively lower propensity for the Cr(III) to bind covalently instead of electrostatically through $Cr(H_2O)_6^{3+}$. Replacing SO_4^{2-} with Cl^- reduces the kosmotropic effect which further favors the electrostatic binding of Cr(III) to collagen. Our findings allow a greater understanding of mechanism-specific metal binding in the collagen molecule. We also report for the first time, surface-enhanced Raman spectroscopy to analyze binding mechanisms in collagen, suggesting a novel way to study chemical modifications in collagen-based biomaterials.

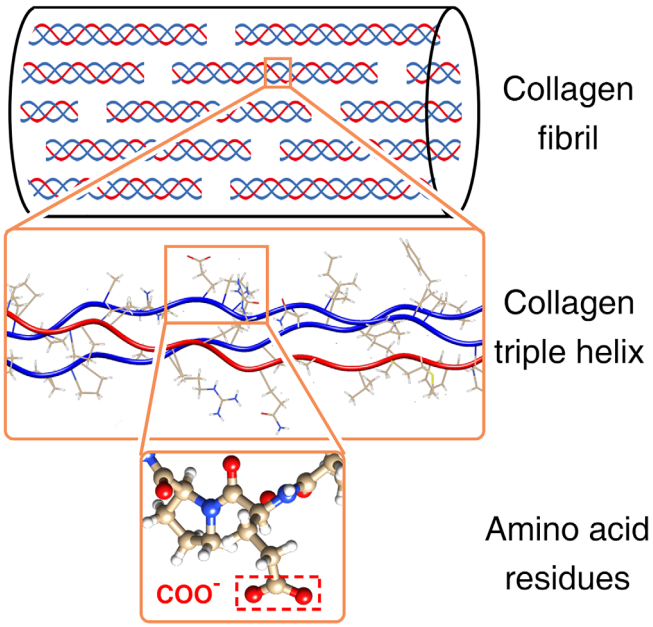
KEYWORDS

chromium, collagen structure, covalent binding, electrostatic interactions, ions

1 | INTRODUCTION

Collagen is the most abundant protein in mammals and provides animal tissues with their structural integrity and stability.[1] Collagenous tissues are composed of fibers and fibrils, followed by the triple helical collagen molecules at the lowest level of its hierarchical organization.[2] These molecules precisely assemble in a quarter-staggered fashion into a fibril, and this supramolecular assembly can be stabilized by covalent crosslinking, metallic binding, hydrogen bonding, and so on.[3–5] In each collagen polypeptide chain, the amino acid sequence exists as repeating Gly-Xaa-Yaa triplets, in which Gly residues are present in the center with low steric hindrance allowing three polypeptide chains to fold into a triple helix.[6] The Xaa and Yaa residues are exposed to the surface of the triple helix and play a crucial role in binding to surrounding collagen molecules to form fibrils,[2,6] or in reacting with crosslinkers and metallic species that alters the structure of native collagen.[5] The changes in collagen structure by metallic species can cause an increase in thermal stability as well as resistance to bacterial attack, similar to organic crosslinkers.[7–9] Among the metallic species commonly used in collagenous tissues, Cr(III) in particular has a unique effect on collagen structure and exhibits properties that may be related to its different reaction mechanism compared to other metal ions.[9,10]

The binding mechanism of Cr(III) with collagen is generally known to proceed by complexing with the COO^- groups of aspartic and glutamic acid (Asp and Glu) residues.[11,12] Later studies revealed the deposition of Cr species that bind with collagen electrostatically.[13–15] However, the preference of Cr(III) to switch between covalent or electrostatic binding with collagen is yet to be understood. Therefore, a comprehensive study of the effect of Cr(III) binding on the different hierarchical levels of collagen, including the role of anions and pH will help us understand its effect on collagen structure. In our study, we treated collagen with chromium sulphate (CS), chromium chloride (CC) and sodium sulphate (SS) at a pH of 2.5 and 4.5 because of the pH-dependent availability of the COO^- groups in collagen. We then followed changes in the hierarchical collagen structure (Scheme 1) using small-angle X-ray scattering (SAXS, for intermolecular structure), differential scanning calorimetry (DSC, for triple helix stability) and vibrational spectroscopies (for secondary structure). Also,



SCHEME 1 Hierarchical structure of collagen from a fibril to the amino acid residues

surface-enhanced Raman spectroscopy (SERS) was used to directly analyze the effect of chemical binding to collagen on its amino acids.

2 | EXPERIMENTAL SECTION

2.1 | Materials

For collagen samples: Dry bovine skin (pH 3); chromium sulfate (25% Cr_2O_3 equivalent), LANXESS; chromium chloride hexahydrate, Sigma-Aldrich; sodium sulfate, Clarks; sodium chloride, Dominion Salt; sodium hydroxide, Sigma-Aldrich.

For silver nanoparticles: silver nitrate, Pure Science; sodium borohydride, Sigma-Aldrich; trisodium citrate dehydrate, Sigma-Aldrich.

2.2 | Preparation of collagen samples

Bovine collagen was obtained by grinding the dry bovine skins into powdery fiber. Then, 0.3 g dry collagen fiber was rehydrated using 0.6 mL 2.5 M sodium chloride (Coll-2.5), then treated using chromium sulfate (Coll-2.5-CS) or chromium chloride (Coll-2.5-CC) at concentrations of 29, 86, 171, and 285 M of Cr per molar of collagen. The final pH of each sample was 2.0 to 2.5.

Also, 0.3 g dry collagen fiber was rehydrated using 1.2 mL of 0.1 M sodium hydroxide, then treated using chromium sulfate (Coll-4.5-CS) or chromium chloride (Coll-4.5-CC) at concentrations of 29, 86, 171, and 285 M of Cr per molar of collagen. The final pH of each sample was around 4.0 to 4.5.

Alternatively, 0.3 g dry collagen fiber was rehydrated using 0.6 mL of 0.1 M sodium hydroxide to a pH of 4.5 (Coll-4.5) as the control. Then, Coll-4.5 were treated using sodium sulfate (Coll-4.5-SS) at concentrations of 44, 129, 257, 428 M of SO_4^{2-} per molar of collagen (equivalent to 29, 86, 171, 285 M of Cr per molar of collagen in chromium sulfate). The final pH of each sample was 4.0 to 4.5.

2.3 | Synchrotron small-angle x-ray scattering

Synchrotron small-angle x-ray scattering (SAXS) measurements were performed on TLS beamline 23A1 at the National Synchrotron Radiation Research Centre (NSRRC) in Hsinchu, Taiwan. Collagen samples were loaded into sample cell of size 0.5 cm × 0.5 cm × 2 mm ($L \times W \times H$) and sealed between polyimide films to prevent sample dehydration. Measurements of 30 seconds with X-ray energy of 15 keV were performed at room temperature (around 25 °C). Scattered radiation was collected using a Pilatus 1 M detector located at a distance of 2.602 m from the sample.

The scattered intensity $I(q)$ is presented as a function of scattering vector, q, with $q = 4\pi\sin(\theta/2)/\lambda$, where θ is the angle between incident and scattered radiation. The acquired SAXS scattering patterns were radially integrated into 1D SAXS profiles, and the diffraction peaks were fitted to calculate the peak areas following previously reported approach.[16,17] The diffraction peaks are modeled as Gaussians,

$$I_{\text{peaks}}(q) = \sum_n \frac{A_n}{w(q)\sqrt{\pi/2}} \exp\left(\frac{-2\left(q-\frac{2\pi n}{d}\right)^2}{w(q)^2}\right)$$

where A_n is the area of peak n, d is the D-period (in Å), and $w(q)$ is the width, expressed as $w(q) = a + bq$, where a and b are fitted parameters. Relative peak intensity of fifth to third was calculated as: $R_{5/3} = A_5/A_3$.

2.4 | Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were carried out using a Q2000 DSC (TA Instruments). The as-prepared collagen samples were encapsulated in hermetically sealed aluminum pans (10 μL) and measured over a temperature range of 30 to 120 °C under N_2 purge and a heating rate of 5 °C/min. Extrapolated onset temperature was presented as denaturation temperature of collagen (T_d) in each sample.

2.5 | Fourier-transformed Infrared spectroscopy

Fourier-transformed Infrared spectroscopy (FTIR) measurements were conducted using iD5 ATR on Nicolet iS5 Spectrometer (ThermoFisher

Scientific) in an attenuated total reflectance (ATR) mode using a crystal zinc selenide as detector. Air-dried collagen samples were directly loaded and measured at a frequency interval of 500 to 4000 cm^{-1}. The number of scanning and resolution were set at 16 and 4, respectively.

2.6 | Synthesis of silver nanoparticles

The synthesis of silver nanoparticles for SERS followed a previous report.[18] A seed solution was prepared by mixing 20 mL of 1% (w/v, same below) sodium citrate solution and 75 mL of water, heated to 70 °C for 15 minutes, followed by the addition of 1.7 mL of 1% silver nitrate solution and then 2 mL of 0.1% freshly prepared sodium borohydride solution. The reaction mixture was vigorously stirred at 70 °C for 1 hour and cooled to room temperature. In the second step, 2 mL of 1% sodium citrate solution was added to 75 mL of water and boil for 15 minutes. Ten milliliter of seed solution and 1.7 mL 1% silver nitrate solution were added and refluxed under vigorous stirring for 1 hour. After that, 2 mL of 1% sodium citrate solution and 1.7 mL 1% silver nitrate solution were added and rigorously stirred for 1 hour and cooled to room temperature. In the third step, 2 mL of 1% sodium citrate solution was added to 80 mL of water and heated to 80 °C for 15 minutes. Ten milliliter solution from the second step was added as seed, followed by 1.7 mL of 1% silver nitrate, and the mixture was kept at 80 °C for 2 hours before cooled down to room temperature. The solutions were stored at 4 °C before used in SERS experiments.

2.7 | Raman spectroscopy and surface-enhanced Raman spectroscopy

Raman and surface-enhanced Raman spectroscopy (SERS) experiments were carried out using a Labram HR spectrometer (Horiba Jobin-Yvon) equipped with a Symphony CCD detector cooled with liquid N_2 at −130 °C, in a back-scattering configuration through a LM Plan FI 50x/NA0.50 Olympus air objective by focusing on the air/solid interface. Collagen samples were mixed with 100 μL of silver nanoparticles and excited using a 514 nm Argon-ion laser (Melles-Griot) at approximately 10 mW with an exposition time of 5 seconds and 5 accumulations. A polynomial baseline correction was done to remove the background scattering from the SERS substrate (Figure S1) and the fluorescence of collagen.

3 | RESULTS AND DISCUSSION

In Figure 1A native collagen fibrils (Coll-4.5, pH 4.5) show characteristic SAXS profiles exhibiting well-resolved diffraction peaks from third to ninth order. The third order peak intensity decreases while the fourth to ninth order peak increases with Cr(III) concentration in Coll-4.5-CS due to the presence of SO_4^{2-}. The changes in peak intensities are linked to the electron density changes in the long-range ordered structure in collagen fibrils.[19] The X-ray scattering length in peptide chains with light elements (H, C, N, O, and S) is lower than metallic ions with high atomic number such as Cr(III),[20] Therefore, if Cr(III) binds to collagen in a long-range ordered fashion, it can contribute to

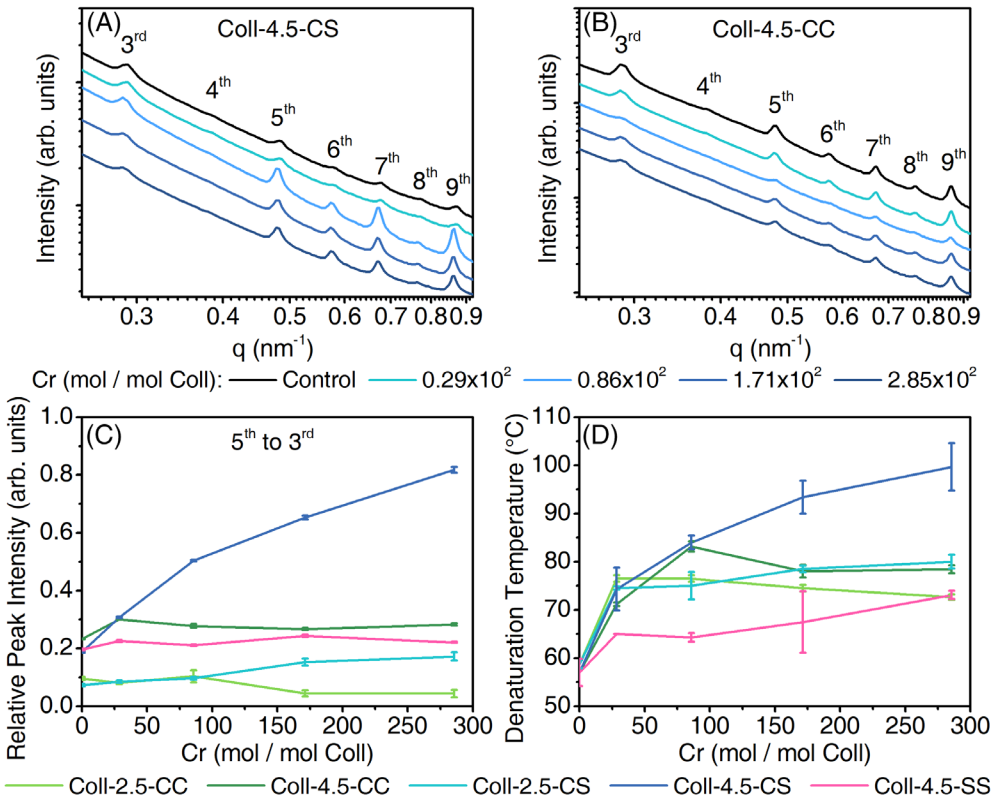


FIGURE 1 A,B, Radially integrated SAXS profiles (stacked) of native collagen at pH 4.5 (black curve) and collagen treated with different concentrations of Cr(III) in the presence of (A) SO_4^{2-} (Coll-4.5-CS) or (B) Cl^- (Coll-4.5-CC). C, Relative intensity of the fifth to third order peak from collagen axial staggering (fitting errors shown), and D, T_d of collagen at different Cr(III) concentration with SO_4^{2-} or Cl^-, at pH 2.5 and 4.5 (Coll-2.5-CS, Coll-2.5-CC, Coll-4.5-CS, and Coll-4.5-CC), measured using DSC (error bars represent SD of the mean, n = 2). Collagen treated with Na^+ and SO_4^{2-} (Coll-4.5-SS) were also shown at concentrations of equivalent SO_4^{2-} in Coll-4.5-CS

the diffraction peak intensities. At pH 4.5, according to the pKa of Asp (3.8) and Glu (4.2) in collagen, the c[COO^-]/(c[COOH] + c[COO^-]) is estimated to be 65%, allowing binding of Cr(III) to collagen via coordinative covalent bonds.[21] Accordingly, there is merely 3% of COO^- at pH 2.5 (Coll-2.5-CS) resulting in lesser binding of Cr(III) and therefore unchanged peak intensities (Figures S2 and S3).

However, when SO_4^{2-} (Coll-4.5-CS) is replaced by Cl^- at pH 4.5 (Coll-4.5-CC), no significant changes in peak intensity were observed at different Cr concentrations (Figure 1B). Cr(III) binds to collagen by forming coordinate covalent bonds with COO^- groups in addition to electrostatic interactions with collagen by means of a solvated aquo complex, $Cr(H_2O)_6^{3+}$.[15] Such electrostatic interactions can be disrupted by other cations in the solution (e.g., Na^+), leading to a less specific positioning of the aquo complexes in the repeating units in collagen fibrils. Since the diffraction peaks can only be affected by changes in the long-range ordered structure,[20] $Cr(H_2O)_6^{3+}$ in collagen tends to have only minor contribution to the diffraction peaks. Instead, covalently bound Cr(III) to the COO^- groups in the periodically staggered collagen molecules can enhance the diffraction peak intensity of X-ray. So, why does changing the anion from SO_4^{2-} to Cl^- cause the collagen molecule to scatter differently? The propensity of the kosmotropic SO_4^{2-} anion to form a hydrogen bond with water can compete with the formation of $Cr(H_2O)_6^{3+}$, and therefore increase the availability of Cr^{3+} to covalently bind to COO^- groups in collagen. In contrast, Cl^- is more chaotropic and thus less solvated than SO_4^{2-}, leading to an increase in the proportion of bulk water favoring formation of $Cr(H_2O)_6^{3+}$.[22,23] This can increase the electrostatic interactions with collagen and decreases the amount of covalent coordinative bonds with COO^- in the equilibrium mixture. The selective mechanism is also highlighted by the relative intensity of the fifth to third order peak (Figure 1C). Previous studies have shown even order peaks to be significantly affected by hydration level and not suitable for analyzing the effect of binding in hydrated collagen.[24,25] From Coll-4.5-CS, an increase in the ratio of the fifth to third order peak was found with the concentration of chromium sulfate, whereas in the absence of either Cr^{3+} (Coll-4.5-SS), SO_4^{2-} (Coll-4.5-CC) or suitable pH (Coll-2.5-CS and Coll-2.5-CC), no significant changes can be found.

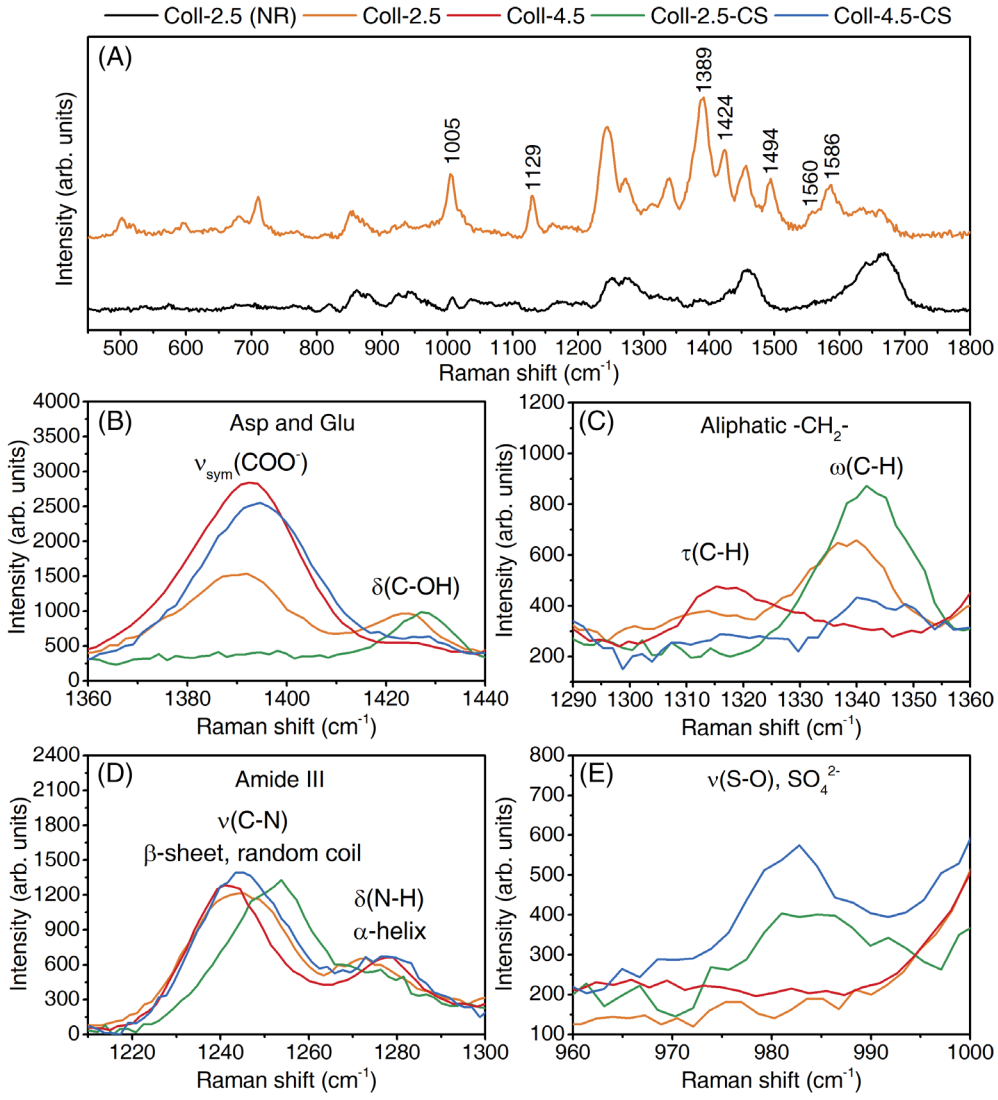


FIGURE 2 A, NR vs SER spectra of native collagen at pH 2.5 (Coll-2.5). B-E, Collagen SER spectra of Coll-2.5, Coll-4.5, Coll-2.5-CS, and Coll-4.5-CS are shown to highlight: B, $\nu_{sym}(COO^-)$ (1389 cm^{-1}) and δ(C-OH) (1424 cm^{-1}); C, τ(C-H) (1311 cm^{-1}) and ω(C-H) (1338 cm^{-1}) from aliphatic —CH_2—; D, amide III (1245 cm^{-1} and 1275 cm^{-1}); E, ν(S-O) from SO_4^{2-} (980 cm^{-1}). Cr concentration = 285 (mol/mol Coll). Baselines of the spectra were corrected to remove background from the SERS substrate and the fluorescence of collagen

Thermal stability or denaturation temperature (T_d) of collagen increases with binding interactions.[5] In Coll-4.5-CS, the highest T_d at 100 °C was observed (Figure 1D) due to the covalent binding of Cr(III)-OOC and from the hydrogen bonding of the kosmotropic SO_4^{2-} via water bridges. The Cl^- in Coll-4.5-CC impedes covalent binding but favors electrostatic interactions from $Cr(H_2O)_6^{3+}$, in addition to the weaker hydrogen bonding effect of the chaotropic Cl^- with water, giving a T_d of only 80 °C. When no Cr(III) is involved (Coll-4.5-SS), a lower T_d of 70 °C was observed from hydrogen bonding of SO_4^{2-}. Covalent binding is generally less favored at pH 2.5 (Coll-2.5-CS or Coll-2.5-CC) due to the decreased amount of COO^- groups in collagen,[26] yet with only the electrostatic interactions from $Cr(H_2O)_6^{3+}$, the collagen showed a T_d at 80 °C.

To focus on the amino acid groups in collagen we conducted SERS in a first attempt to directly examine the covalent binding in collagen (Figure 2). When an exciting laser beam hits an amino acid group that is in the vicinity of the surface of the colloidal silver nanoparticles, enhanced Raman scattering intensity from the corresponding group is observed.[27] Figure 2A shows a comparison between normal Raman (NR) and SER spectra of Coll-2.5: characteristic amide bands are observed in both,[28] but enhancement on the silver surface resolves features from specific amino acid groups (Table S1).[28–33] The potential interferences from citrate-related bands are ruled out by the highly consistent SER spectrum of collagen enhanced by a citrate-free, borohydride-reduced silver nanoparticle (Figure S4). Distinct bands at 1586, 1494, and 1005 cm^{-1} in collagen SER spectra are attributed to the ring breathing modes of phenylalanine (Phe) or tyrosine (Tyr) residues.[30,31] A sharp band from the rocking of NH_3^+ group is also resolved at 1129 cm^{-1}.[30,34] More importantly, the strongest band at 1389 cm^{-1} as well as the adjacent band at 1424 cm^{-1} and a shoulder at 1560 cm^{-1} are related to the COO^- or COOH group in Asp and Glu residues and are assigned to $\nu_{sym}(COO^-)$, δ(C-OH) and $\nu_{asym}(COO^-)$, respectively.[35] As shown in Figure 2B, changes in pH from 4.5 to 2.5 (Coll-4.5-Coll-2.5) results in a halving of the intensity of the $\nu_{sym}(COO^-)$ band and an increase in δ(C-OH) band.[30–32] The estimated amount of COO^- in collagen at pH 4.5 is 65% but when acidified, it decreases to 3% at pH 2.5, showing a nonlinear correlation with the SERS intensity. Collagen contains, on average, 95 Asp and Glu residues in every 1000 residues, that is, 62 mol of COO^- sites per mol of collagen at pH 4.5.[21,36] When we add as high as 285 mol of Cr(III) to each mol of collagen (Coll-4.5-CS), it should be more than sufficient to occupy all the COO^- groups. However, comparing Coll-4.5-CS with Coll-4.5 (Figure 2B), minimal change was found in the $\nu_{sym}(COO^-)$ band, suggesting that an adequate amount of the COO^- groups in collagen remain unbound and that the electrostatic binding mechanism plays a dominant role. Covalent binding, although minimal, is confirmed by a significant decrease in the $\nu_{sym}(COO^-)$ and $\nu_{asym}(COO^-)$ band when Cr(III) is introduced at pH 2.5 (Coll-2.5 vs Coll-2.5-CS, Figure 2B and Figure S4). Also as shown Figure 2C, the aliphatic τ(C-H) and ω(C-H) bands in Coll-4.5-CS were found to be similar to Coll-2.5 instead of Coll-4.5, indicating covalently bound COO^- in the collagen.[28,30,33] Additionally, although the ratio of the intensity of the amide III bands in collagen remains unchanged (Figure 2D, I_{1245}/I_{1275}), a shift of the 1245 cm^{-1} band is observed from 1240 cm^{-1} in Coll-4.5 to 1245 cm^{-1} in Coll-2.5 and Coll-4.5-CS, and then to 1250 cm^{-1} in Coll-2.5-CS, indicating variations in the secondary structure of collagen.[28,29,37] In Coll-2.5-CS, the COO^- groups are covalently bound with Cr(III) and hydrogen to an overall higher degree than the others, therefore leading to changes in the interactions between polypeptide chains affecting the secondary structure of collagen accordingly. The ν(S-O) from SO_4^{2-} is also observed in Coll-2.5-CS and Coll-4.5-CS, confirming its presence (Figure 2E, Figure S5).[38,39] Both observations are also confirmed using FTIR spectra (Figure S6).

4 | CONCLUSIONS

In summary, we described an anion-regulated binding mechanism of Cr(III) in collagen based on structural changes using SAXS and SERS. Both covalent and electrostatic bindings coexist, and an increase in the kosmotropicity of the anion (SO_4^{2-} > Cl^-) promotes the covalent binding of Cr(III) to collagen. This is the first report of SERS to analyze binding mechanisms in collagen and opens new pathways to study chemical modifications in collagen-based biomaterials.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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Supporting Information

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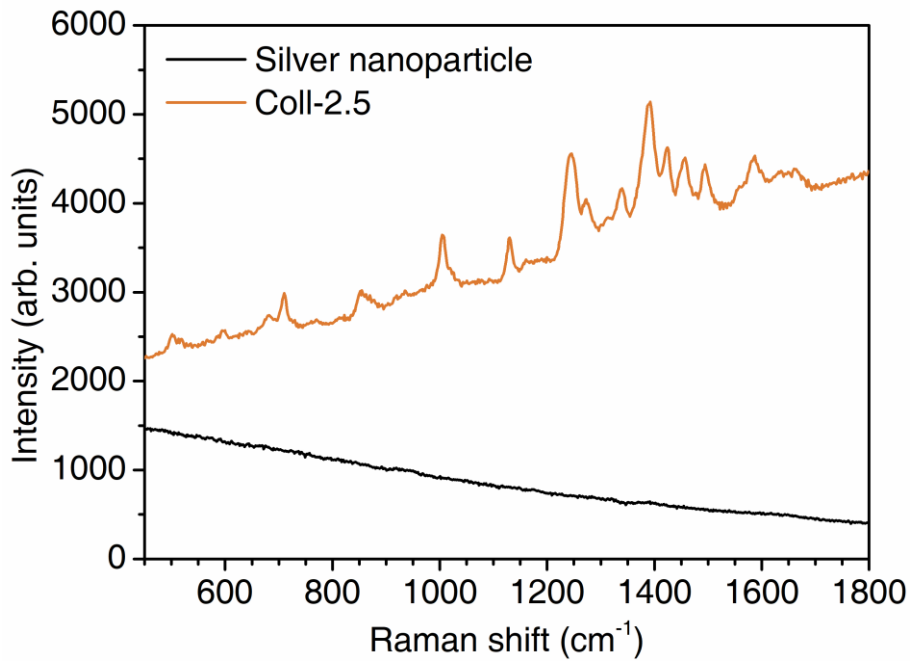

Figure S1. Raman spectrum of the SERS substrate (silver nanoparticle) and SER spectrum of Coll-2.5 before baseline correction.

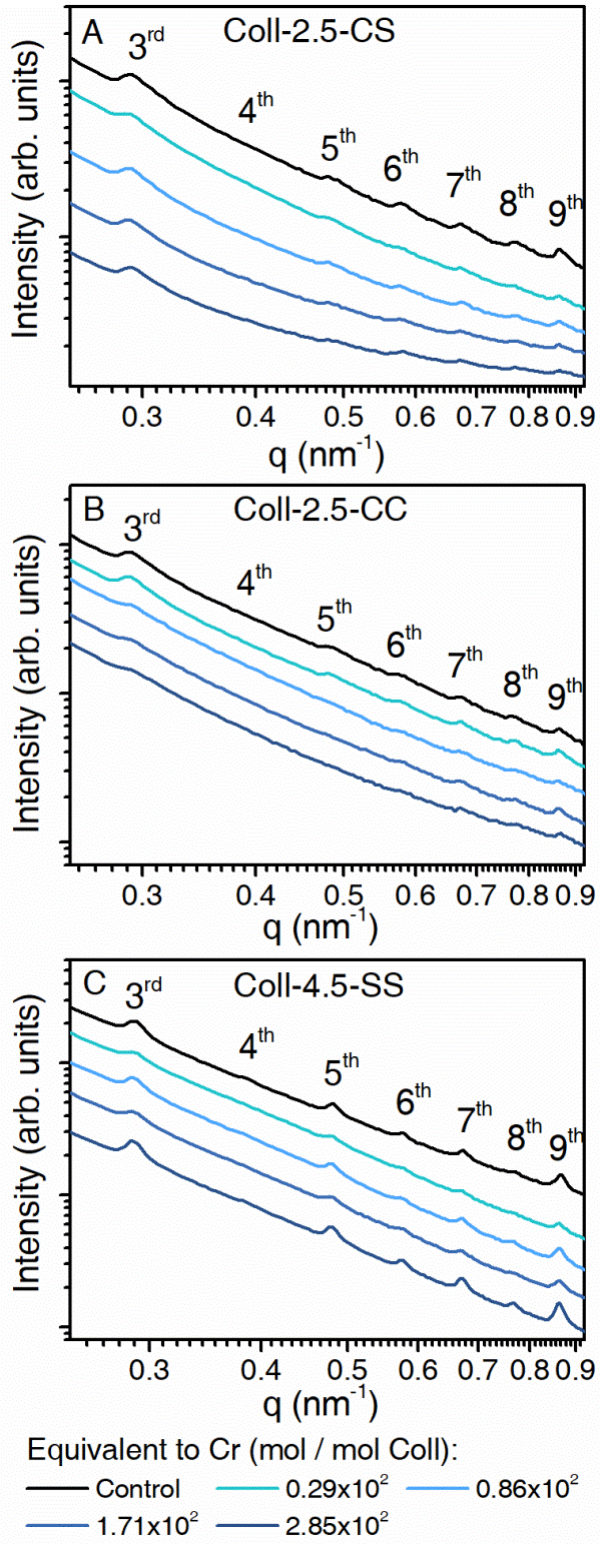

Figure S2. Radially integrated SAXS profiles of (A) Coll-2.5-CS, (B) Coll-2.5-CC and (C) Coll-4.5-SS at different concentrations.

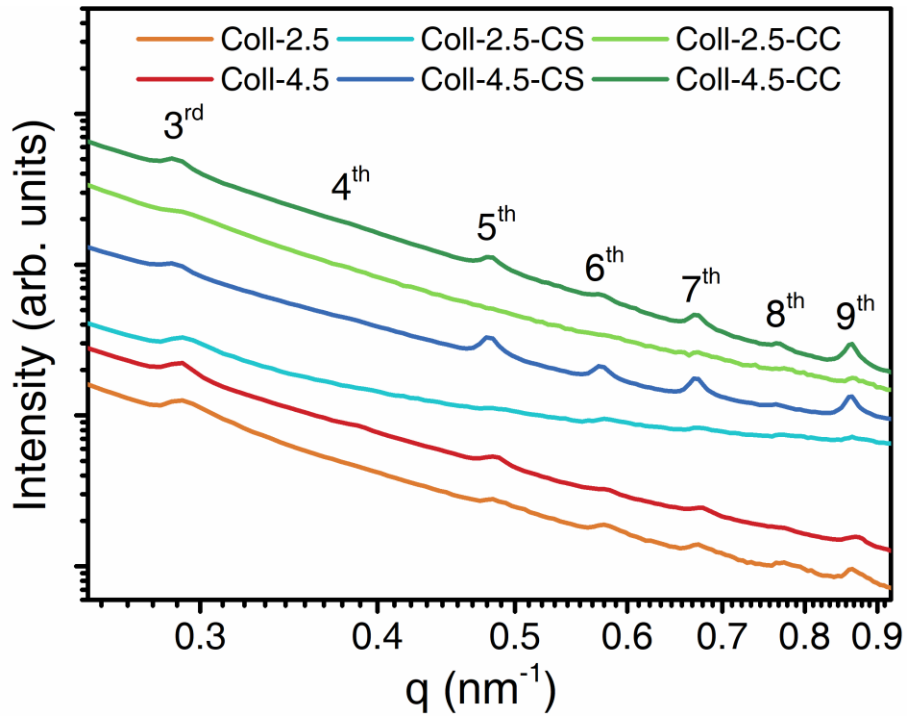

Figure S3. Representative SAXS profiles of Coll-2.5, Coll-4.5, Coll-2.5-CS, Coll-4.5-CS, Coll-2.5-CC and Coll-4.5-CC, showing the relative intensity of the 3rd order peak decreases in Coll-4.5-CS, compared to the other samples. Cr concentration = 285 (mol / mol coll).

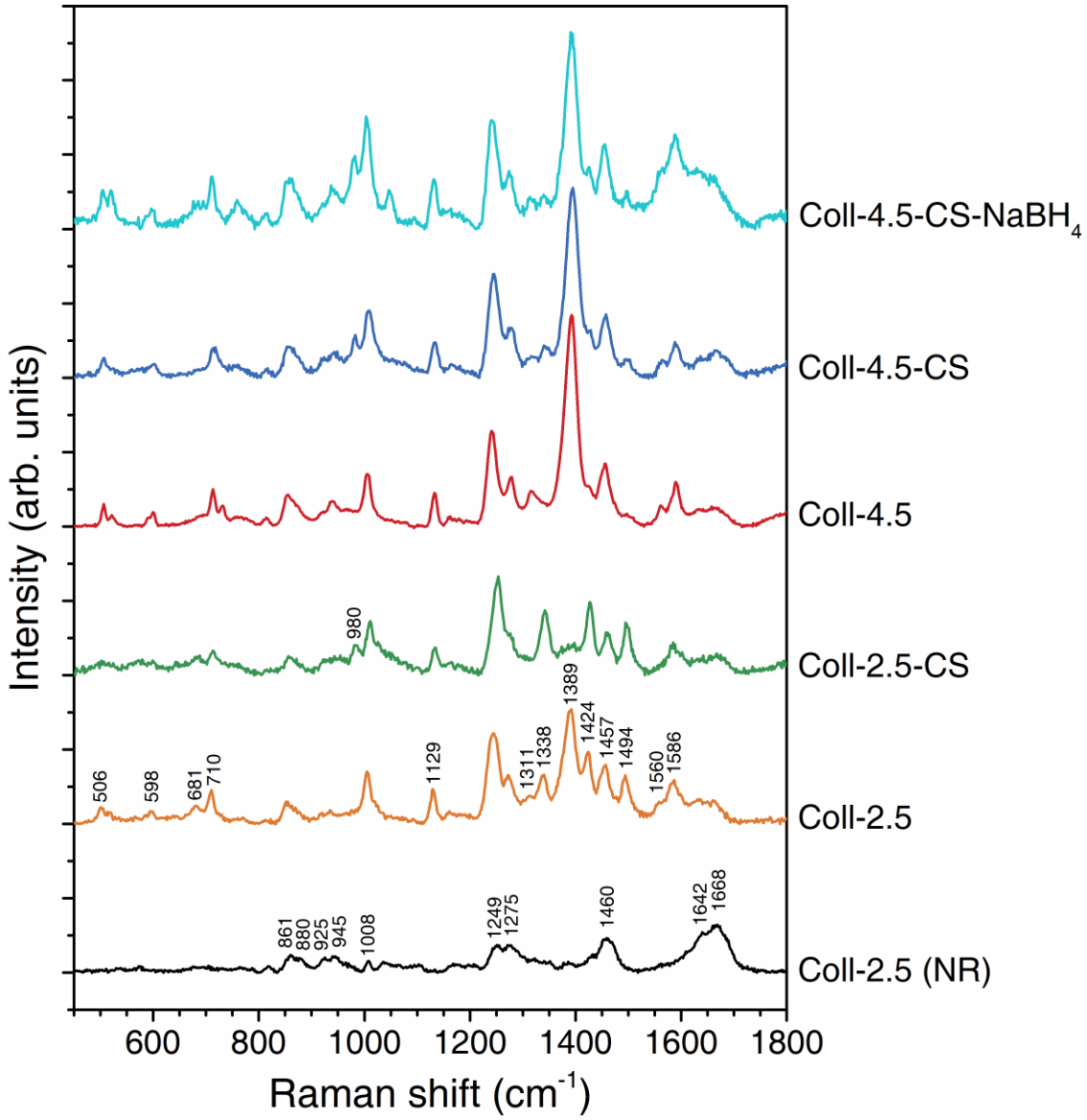

Figure S4. NR spectra of Coll-2.5 and SER spectra of Coll-2.5, Coll-4.5, Coll-2.5-CS and Coll-4.5-CS ranging from 450 cm^{-1} to 1800 cm^{-1}. Cr concentration = 285 (mol / mol coll). All those SER spectra were measured using citrated-reduced silver nanoparticle prepared using the method mentioned in the main text. Coll-4.5-CS-$NaBH_4$ (light blue) is a SER spectrum enhanced using sodium borohydride reduced silver nanoparticle[1], showing high consistency with Coll-4.5-CS (dark blue) enhanced using citrate-reduced silver nanoparticle. This evidence rules out the potential interference from citrate-related bands in collagen SER spectra.

Table S1. Assignment of peaks in Raman and SER spectra of Coll-2.5.

Raman	SERS	Assignment*	Reference
1668	1662	ν(C=O), amide I, α-helix and β-sheet	[2, 3]
1642	1637	ν(C=O), amide I, 3_{10}-helix	[2, 3]
	1586	Hyp; Ring vibration (ν_{8b}), Phe or Tyr	[3, 4]
	1560	$\nu_{asym}(COO^-)$	[5, 6]
	1494	$\delta(CH_3)$; Ring vibration (ν_{19a}), Phe or Tyr	[3, 6]
1460	1457	δ_s(C-H), aliphatic -CH_2-	[4]
	1424	δ(C-OH)	[7]
	1389	$\nu_{sym}(COO^-)$	[3-5, 8]
	1338	ω(C-H), aliphatic -CH_2-	[2, 4, 9]
	1311	τ(C-H), aliphatic -CH_2-	[2, 4, 9]
1275	1273	δ(N-H), amide III, α-helix	[10-12]
1249	1246	ν(C-N), amide III, random coil	[10-12]
	1129	$\rho(NH_3^+)$	[4, 13]
1008	1005	Ring breathing (ν_1), Phe	[3, 4]
945	935	ν(C-C), protein backbone	[2]
925	920	ν(C-COO^-), Asp or Glu; ν(C-C), Pro ring	[2, 4, 9]
880	870	ν(C-C), Hyp ring	[2, 9]
861	855	ν(C-C), Pro ring; Ring breathing (ν_1), Tyr; $\delta(COO^-)$	[2, 9, 14-16]

818	814	ν(C-C), collagen backbone	[17]
	710	δ, ω(COO^-); ν(C–S), Met	[5]
	681	ν(C–S), Met	[4]
	598	ρ(CO), Pro	[5]
	521	δ(C-C-N), Pro	[5, 18]
	506	Collagen backbone deformation	[18]

*ν = stretching; δ = bending; ρ = rocking; ω = wagging; τ = twisting.

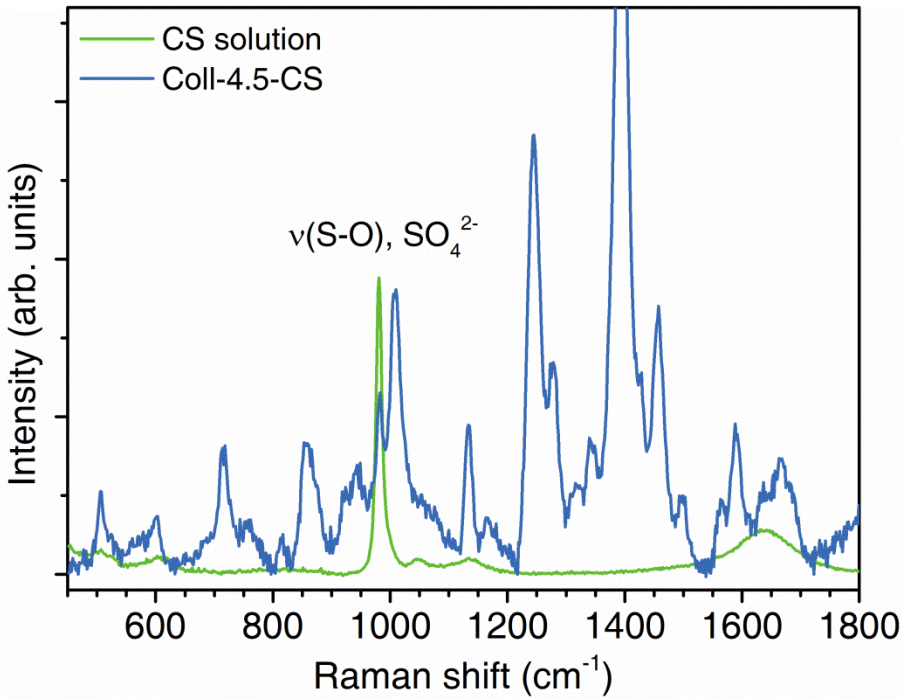

Figure S5. SER spectrum of Coll-4.5-CS (Cr concentration = 285 (mol / mol coll) and Raman spectrum CS solution, highlighting the peak at 980 cm^{-1} (ν(S-O) from SO_4^{2-}).

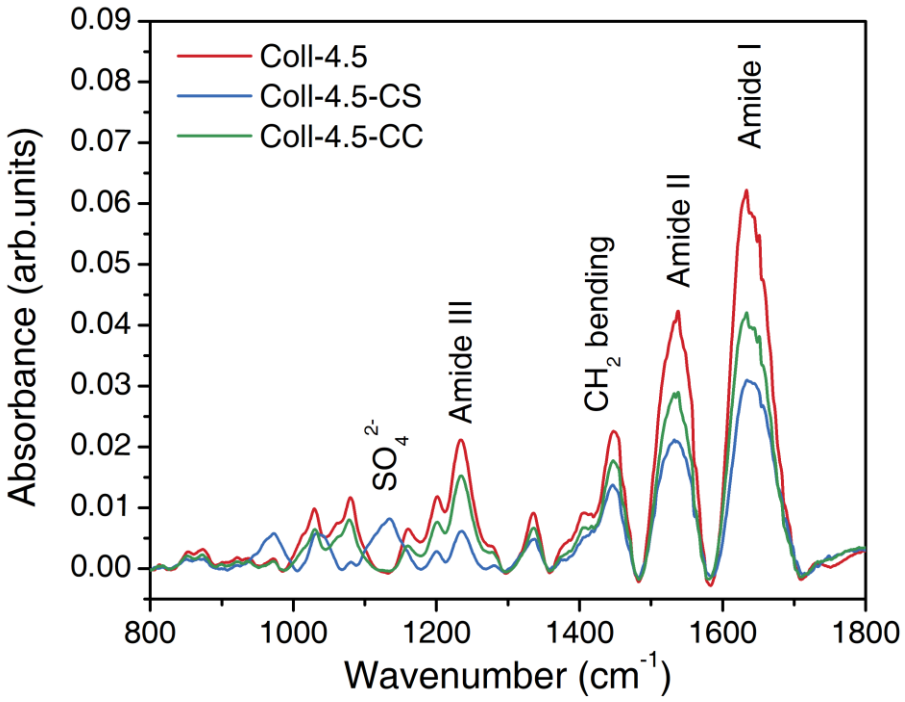

Figure S6. FTIR spectra of native collagen at pH 4.5 (Coll-4.5) and collagen treated with Cr(III) in the presence of SO_4^{2-} (Coll-4.5-CS) and Cl^- (Coll-4.5-CC). Cr concentration = 285 (mol / mol coll).

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