Molecular Interactions Between Type I Collagen and Metal Complex: From Computational Modelling to Experimental Characterisation

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Abstract

Computational modelling was used to construct the models of type I collagen and the Zr-Al-Ti complex, as well as simulating the interactions between them. Hydrogen bond and Van der Waals' forces exist between collagen and the Zr-Al-Ti complex. Furthermore, other bonding regions between central metal ions and O, N, H atoms were calculated. Furthermore, experimental characterisations were carried out to better verify the results of computational modelling, results showed that the spectra of FTIR, UV-DRS and fluorescence were all shifted to some extent. Specifically, UV-DRS and fluorescence detection suggested that the alteration appeared around aromatic residues, tyrosine and phenylalanine. Further, XPS proved that the N atom showed a higher possibility of coordinating with the metal complex than did O. Accompanied with DSC and TG detections, it has been confirmed that the interactions brought by Zr-Al-Ti complex gave rise to the increase of denaturation temperature of collagen from 66.51°C to 88.1°C. In addition, results obtained from SEM and AFM show It is obvious that the original collagen fibre has been changed into a tight layer by layer structure, and it could be observed that metal complex particles filled in between collagen fibres ,this together with strong chemical bonding between them, finally stabilized the structure of collagen.

摘要：将计算建模用于构建I型胶原和ZrAlTi配合物的模型，并模拟两者之间的相互作用。结果表明，胶原与ZrAlTi配合物之间不仅存在氢键和范德华力，配合物金属中心离子与胶原分子中的O、N、H原子还有其它键合力存在。FTIR、UVDRS和荧光光谱均显示在一定程度上都有所偏移，UVDRS和荧光检测表明芳香残基、酪氨酸和苯丙氨酸周围出现改变。XPS证明N原子与ZrAlTi配合物的配位的可能性更高。DSC和TG检测表明，ZrAlTi配合物与I型胶原分子相互作用使胶原分子变性温度从66.51℃升高到88.1℃。SEM和AFM结果显示，原胶原纤维层层堆积，金属络合物颗粒填充在胶原纤维中使ZrAlTi配合物与I型胶原分子产生了强烈化学键合，从而提高了胶原分子结构稳定性。实验结果较好地验证了计算建模的结果。

1 INTRODUCTION

Type I collagen forms more than 90% of the organic mass of bone and is often extracted from tendons, skin, ligaments, cornea and cartilage. Type I collagen has been confirmed to have great biocompatibility, biodegradability, low immunogenicity and is capable of promoting the growth and proliferation of cells.[1-3] There are great possibilities for type I collagen to be applied to widespread industrial use in medicine, cosmetics and food industry. Specifically, being used as a biomaterial has attracted many researchers to conduct deep studies of collagen.[4-6] However,natural type I collagen without any treatment has weak strength and poor storage stability. It is hardly able to offer enough support, especially when being used as a scaffold biomaterial. Consequently, it is essential to improve the mechanical stabilities and collagenase resistance by introducing suitable cross-linking in collagen. A number of researches relating to collagen stabilization with various cross-linkers have been carried out with a long history. Also metal complexes used as a kind of cross-linking agent have been researched for centuries.

So far, chrome tanning still occupies the global leather industry's leading position. However, at the beginning of the twentieth century, the potential problems caused by chromium (VI) have concerned environmentalists. Since then, the harm caused by chromium to humans and the environment has received wide attention. Other metal complexes such as zirconium, aluminum, titanium and iron are less toxic and are considered to be possible substitutes for chromium.

Early in 1893, A. Werner put forward 'coordination theory'. Since then, metal complexes have been widely used in biomaterials, drugs, catalysts, luminescent materials and magnetic materials.[7] Group 4 transition metal complexes have continuously attracted interest owing to their low or no toxicity and low cost. Titanium complexes can catalyse many important reactions, while traditionally zirconium complexes have been less frequently employed. The major advantages of titanium and zirconium are the high abundance of

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these elements and the possibility of adjusting reactivity and selectivity by use of ligands.[8] It is known that metal ions can interact with proteins *via* coordinating with amino acids in side chains. Metal complexes with labile ligands that can be exchanged offer control in the specificity of the coordination interactions through the geometric preferences of the metal ion and shape of the compound imposed by the retained ligands.[9]

However, the structure and composition of the Zr-Al-Ti complex are far more complicated than that of single metal complex,[10] the mechanism of interaction with collagen still remained unknown. It is of great importance to acknowledge interaction modes between collagen and Zr-Al-Ti complex, which might be instructive to generate new tanning agents, drugs and materials.

2 EXPERIMENTAL PROCEDURES

2.1 Reagents and chemicals

Natural type I collagen was prepared according to our previous work. Briefly, porcine acellular dermal matrix provided by Jiangyin Benshine Biological Technology Co. Ltd., was first cut into pieces. A certain weight of porcine acellular dermal matrix was soaked in Tris-HCl buffer (pH7.4) for 2 hours at 4°C. Then a defined volume of acetic acid (pH2.2) was used to soak the sample for another 2 hours, adding 2% pepsin (1:3000) and stirring for 26 hours at 4°C. After centrifugation, pH was then adjusted to 7.5. Along with the addition of $(NH_4)_2SO_4$, collagen was slowly salted out after standing for 10~12 hours. Through dialysis and lyophilization, the preparation of Type I collagen was completed. The Zr-Al-Ti metal complex was provided by Jinkun Chemical Co. Ltd. Other related chemicals were all of analytical grade.

2.2 Computational modeling

2mol/L Zr-Al-Ti complex solution was heated to 90~100°C for 0.5~1.0 hours, cured for a while and stored at 4°C in the refrigerator to produce the final crystals. Zr-Al-Ti complex crystals were then applied to X-ray diffraction pattern detection. The collected data were used for the computer simulation.

The Reflex Plus and Reflex modules of Materials Studio (Accelrys MS 5.5) were applied to calculate and simulate the crystal structure of Zr-Al-Ti complex. Briefly, XRD data was introduced first, and then indexing, Pawley fitting, structure solution and Rietveld refinement were applied respectively. Finally, the structure was basically constructed.

The module of Build in MS was first used to construct the Pro-Hyp-Gly unit. The conformation of the unit was then optimised in COMPASS by using the module Minimizer of Discover. And on this basis, the $3(Pro\text{-}Hyp\text{-}Gly)_{10}$ model was optimised to achieve the best triple helical units.

In addition, an individual helix α chain was set as the polypeptide model to calculate the hydrogen bond and close packing with the tool, calculating the hydrogen bonds of MS. Specifically when calculating the close packing, the radii of VDW, Slater, Metallic, Ionic and covalent were recognised. Repeatedly changing the site of the polypeptide while the position of the complex was fixed, the hydrogen bond and close packing were observed.

2.3 Optimisation of reaction between collagen and Zr-Al-Ti complex

2mg/mL collagen solution was firstly prepared by dissolving lyophilized collagen in acetic acid (0.5mol/L). Then 6 groups of collagen solution (50mL) were used to carry out the reaction. Various dosages of Zr-Al-Ti complex 1.0g, 1.5g, 2.0g, 2.5g, 3.0g and 3.5g were added to each group of collagen solution. After shaking for 3 hours in water bath at room temperature, pH was increased to 4.0 by adding $NaHCO_3$ solution (5%), and reaction temperature was elevated to 40°C. After storing for 12 hours, the collagen specimens were then rinsed thoroughly and lyophilized. Each collagen sample was applied to characterisation to find out the optimum dosage of Zr-Al-Ti complex. Afterwards, the optimised group of collagens treated by Zr-Al-Ti complex was applied to further analysis. In an attempt to confirm the optimum dosage of Zr-Al-Ti complex, the free amino and carboxyl content tests were carried out.

2.4 Free amino content

The ninhydrin reaction was performed to determine the free amino content. The modification degree was defined as:

$$\text{Modification degree (\%)} = (NH_0 - NH_t) / NH_0 \times 100$$

Where NH_t and NH_0 represent free amino content after treated and before treated. For each sample, 5 groups of tests were carried out to gain the average value.

2.5 Free carboxyl content

Free carboxyl content could be used to determine the degree of reaction of the Zr-Al-Ti metal complex with the carboxyl groups in collagen. In the present research, conductance titration was applied. Briefly, specified weights of samples and 20mL HCl solution (pH3.0) were put in 100mL flasks and stored for 30 minutes.The flasks were sealed with cellophane, and the conductivity meter was then applied to detect any change in the solution by adding NaOH solution (0.04808mol/L) under a nitrogen flow. Free carboxyl content was obtained according to the equation:

$$\text{Free carboxyl content (\%)} = [(C\Delta V \times 45)/10^6 W] \times 100$$

In which C and ΔV are the concentration of NaOH solution and the volume of NaOH used during titration, respectively. W represents the weight of sample.

2.6 Fourier transformed infrared spectroscopy (FTIR)

1~2mg lyophilised samples were mixed with KBr. The compressed tablets were applied to FTIR analysis (Nicolet iS10, Thermo Scientific Co., America). All

spectra were recorded 32 times at 4000~400cm^{-1} at room temperature under humidity around 65%.

2.7 Diffused reflectance spectrum (UV-DRS)

UV-DRS of samples were determined by UV/VIS/NIR spectrophotometer (UV-3600, Shimadzu Co., Japan). BaSO4 was used as reference. The samples were scanned at the rate of 200nm/min in the range of 200~800nm.

2.8 Fluorescence spectrum

Samples were dissolved in acetic acid solution (0.05mol/L) to prepare collagen solutions with a concentration of 0.1mg/mL. Different collagen solutions were applied to fluorescence spectrophotometer (Hitachi F-4010, Tokyo, Japan) with an excitation wavelength between 240~350nm to define the feature excitation wavelength. The slits of excitation and emission were both set to 5nm for both excitation and emission. The scanning rate was controlled at 120nm/min. the emission spectra were recorded around 280~350nm at the feature excitation wavelength. All the measurements were taken in triplicate.

2.9 X-ray photoelectron spectroscopy (XPS)

Lyophilised samples were analysed by XPS on an Escalab (XSAM800, Kratos Co., England) with Mg-Kα X-ray source (hv=1253.6eV). The binding energy of each element was corrected with C1s (BE=284.7eV). Data handling was analysed by XPSPEAK41 (based on Gaussian-Lorentzian).

2.10 Differential scanning calorimetry (DSC) and Thermogravimetry (TG)

Lyophilised samples (3~5mg) were used to characterise thermal stability by differential scanning calorimeter (DSC-200PC PHOX, Netzsch, Co., Japan). The samples were sealed in aluminum cells with the reference of an empty aluminum pan. The tests were then carried out in the range of 15~180°C with a heating rate of 10K/min under a nitrogen flow 20mL/min. Each measurement was performed at least three times.

Thermal weight loss of each sample (2~5mg) was recorded at TG analyzer (TG 209 F1, Netzsch, Co., Japan) from temperature 40°C to 800°C at a heating rate of 10k/min under nitrogen flow 20mL/min.

2.11 Scanning electron microscopy (SEM)

Cross-section morphology of samples was observed on SEM (S3000N, Hitachi Co., Japan) after gold sputtering. The accelerating voltage was 20kV.

2.12 Atomic force microscopy (AFM)

10μg/mL collagen solutions were prepared by dissolving collagen in acetic acid solution (0.05mol/L). Then each sample solution was dropped on the mica plate and examined by AFM (Shimadzu SPM-9600, Japan) after air drying for 4 hours.

3 RESULTS AND DISCUSSION

3.1 Computational modeling

Materials studio (MS) was firstly applied to construct the crystal structure of Zr-Al-Ti, and then to reflect interactions between collagen and the Zr-Al-Ti complex.

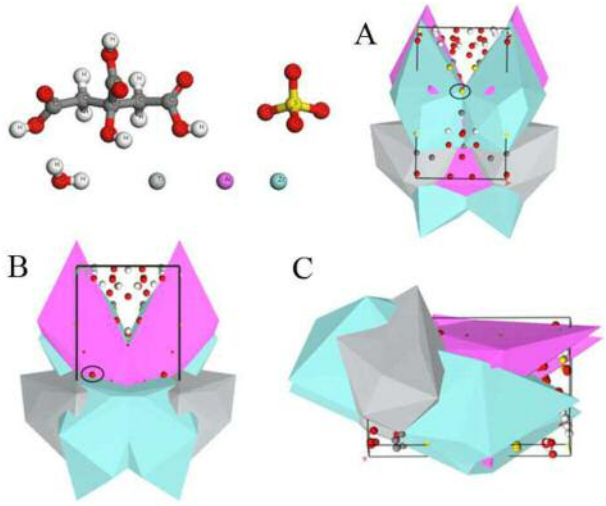


Figure 1. The components of Zr-Al-Ti complex.

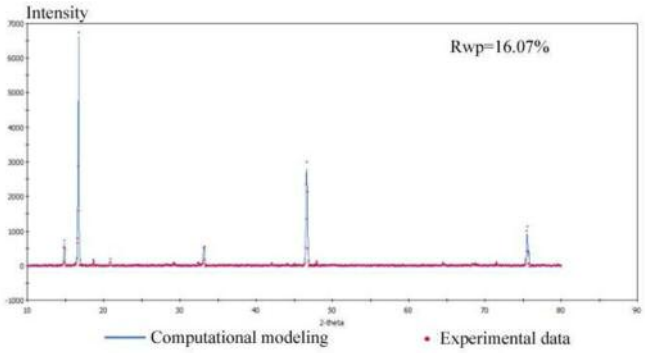


Figure 2. XRD of the Zr-Al-Ti complex.

The three kinds of crystals in Figure 1 have a symmetrical structure, and the metal can well be attached to the corresponding coordinating atoms to form a polyhedron. It can be seen clearly from Figure 1A that atom S (yellow ball) acts as the bridged linkage between Zr ligands, while atom O (red ball) acts as the bridge linkage between Zr and Al. As a whole, the polyhedron complex formed by Zr, Al and Ti embedded to each other, is a complicated compound bonded by covalent bonds, ionic bonds, coordinate bonds, metallic bonds and hydrogen bonds. In addition, part of Al complex was wrapped inside of the crystal by the Zr and Ti complex, binding the complex together and maintaining its stability. Figure 2 shows that the simulated pattern is well fitted with the experiment result, and basically has the same peaks and intensity of XRD which further confirms that the simulated unit cells are consistent with those of the experimental group. Particularly, the value of RWP, which is to evaluate the fitting effect (the former is 4.00:5.00:1.00, the latter is 3.92:4.96:1.00), remained stable after

repeated fine adjustment by Rietveld, showing a good fitting effect, which reveals that the simulated model approaches to its real state.

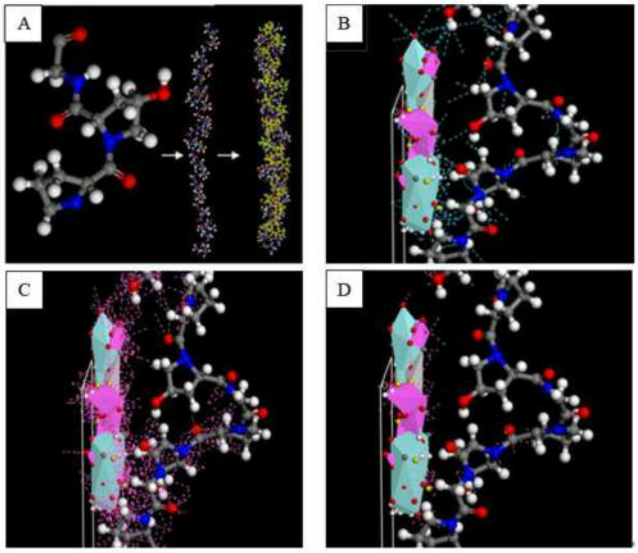


Figure 3. Molecular simulation of interactions between collagen and Zr-Al-Ti complex (Gray ball: C, Red ball: O, White ball: H, Blue ball: N; Blue dotted line (B): Hydrogen bond; Pink dotted line (C, D): Close packing).

It can be seen from Figure 3 that the three-dimensional structure of the polypeptide changed obviously during the process of triple helical structure formation. In the process of energy minimisation, it stretched out to achieve the optimum conformation state. The hydrogen bond cannot be directly calculated by COMPASS in MS, but could be obtained by comprehensively calculating the Van der Waalsí force and electrostatic interaction. The total potential energy of $3(Pro\text{-}Hyp\text{-}Gly)_{10}$ is 10614.22kcal/mol and non-bond energy is 2416.41kcal/mol, which is the sum of Van der Waals' forces and electric potential energy.

In Figures 3B, 3C and 3D, hydrogen bond and Van der Waals' forces exist between collagen and the Zr-Al-Ti complex, and there are also some other bonding regions like Zr-O, Al-O, Ti-O, Zr-N, Al-N, Ti-N, Zr-H, Al-H, Ti-H and so on. It can be preliminarily concluded that Zr-Al-Ti complex can react with the amino group, carboxyl group, hydroxyl group and acylamino group in collagen.

3.2 Modification degree

As referred to earlier,[9] the outer electronic structure and ionic radii of metals differ from each other, as well as the difference of charge on the electron, which defining them as different states in solution. When at pH<3 ,the $-NH_2$ of collagen attracts protons to form $-NH_3^+$, and the Zr complex still holds strong absorbability to collagen. It demonstrates that Zr (IV) mainly reacts with amino groups. Since the electronic structure of Ti (IV) is similar to that of Zr(IV), it has been speculated that Ti (IV) also primarily reacts with amino groups of collagen. However, the reacting site of Al (III) is mainly on carboxyl groups. It is known that -COOH and $-NH_2$ play the main roles in stabilising collagen when reacting with a metal complex.[11] The degree of modification characterised by free amino and carboxyl content are shown in Figure 4 as the 'black downwards triangle' curve and 'star' curve respectively. It is obvious that with the increasing dosage of Zr-Al-Ti complex, the modification degree rose sharply until the ratio of collagen/metal complex reached 1:20~1:25. The modification degree referring to free amino content and carboxyl group remained stable or even decreased afterwards. Notably, amino modification degree could reach 60% around while that of carboxyl content only was 25% at the highest. Zr(IV) and Ti(IV) might account for the absorption of amino groups while Al(III) is responsible for the absorption of carboxyl groups.

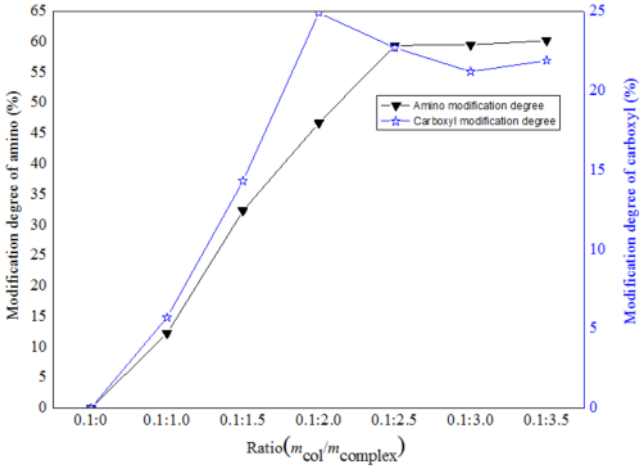


Figure 4. Degree of modification of collagen treated by Zr-Al-Ti complex.

3.3 Spectrum analysis

It is shown in Figure 5 that the amide A of type I collagen blue shifted from 3341.49cm^{-1} to 3421.89cm^{-1} after reacted with Zr-Al-Ti complex and amide B almost disappeared. These alterations of FTIR spectra might be the results of various interactions between collagen and Zr-Al-Ti complex. By introducing Zr-Al-Ti complex, the intermolecular hydrogen bonds at the site of atom N were broken down to some extent, leading to the increase of the force constant of N-H, therefore the absorption frequency blue shifted. It has been suggested[13] that there are generally three different Zr(IV) complexes in solution: negatively charged Zr complex, positively charged Zr complex and electro-neutral Zr complex. At a lower pH, $-NH_2$ of collagen turned into $-NH_3^+$ through protonation, and the $-NH_3^+$ in collagen effectively attracted negatively charged Zr complexes to further interact with each other. Further, the Ti(IV) complex shares the same interaction mode as Zr(IV). Strong bonds shaped to stabilise collagen, resulting in the blue shift of amide A and disappearance of amide B. As for amide I, amide II and amide III, which have usually been used to observe the integrity of the triple helices of collagen, there was no obvious shift except that the amide I red shifted from 1658.30cm^{-1} to 1639cm^{-1}. This red shift is likely due to new hydrogen bonds formed at the -COOH in collagen. Furthermore, atom O could provide lone pair electrons to the Al(III)

complex to form more stable coordination bonds. In that way, polarity of C=O increased, consequently the force constant of C=O decreased and the absorption frequency red shifted.

According to the theory: transition probability = $(4\pi^2/h^2)|\mu_{ab}|^2E_0^2t$, transition probability is defined by transition dipole moment. Atom O and N in amide groups could both offer lone pair electrons to interact central metal ions in Zr-Al-Ti complex, hence the molecular polarity is reduced. This molecular polarity reduction gave rise to the transition dipole moment. Therefore, absorption strength around amide I, II and III decreased. This is also consistent with the results of computational modelling.

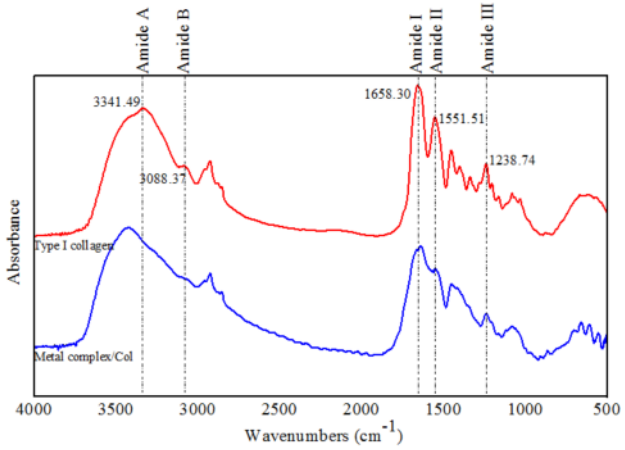


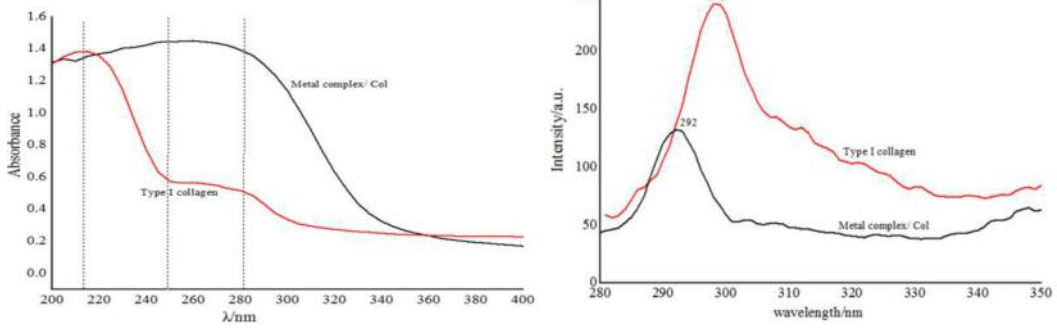
Figure 5. FTIR spectra of Type I collagen treated with Zr-Al-Ti complex.

Figure 6 shows the weak absorption in the range of 250~280nm is caused by the aromatic residues, tyrosine and phenylalanine. It has been suggested that this weak absorption might due to the *n*-π* transition of -C=O.[12] Since tyrosine and phenylalanine both contain lone pair electrons, the electrons showed great capability for coordinating with central metal ions. Thus, the original *n*-π* transition did not occur. The weak absorption around 270nm in UV-DRS disappeared.

In the present study, at the maximum excitation wavelength around 270nm, the feature emission wavelength of tyrosine was detected around 298nm. After treated with the Zr-Al-Ti complex, the feature absorption peak blue shifted from to 292nm and the absorption strength decreased sharply. This change is directly related to the polarity alteration of the environment around tyrosine in solution. It is consistent with the result of UV-DRS spectra.

3.4 XPS analysis

From high resolution spectra in Figure 7, it is clear that the binding energy related to C, O and N changed. Individual binding energy assignments are exhibited in Table III. By reacting with Zr-Al-Ti complex, the basic XPS spectra shapes were retained, but the intensities were decreased to some extent, especially that of O1s and N1s. Central metal ions coordinated with O and N through attracting the lone pair electrons, which is consistent with the results of FTIR, UV-DRS and fluorescence analysis. Peculiarly, the difference of N1s



a. UV-DRS spectra

b. Fluorescence spectra

Figure 6. Spectra of Type I collagen treated with Zr-Al-Ti complex.

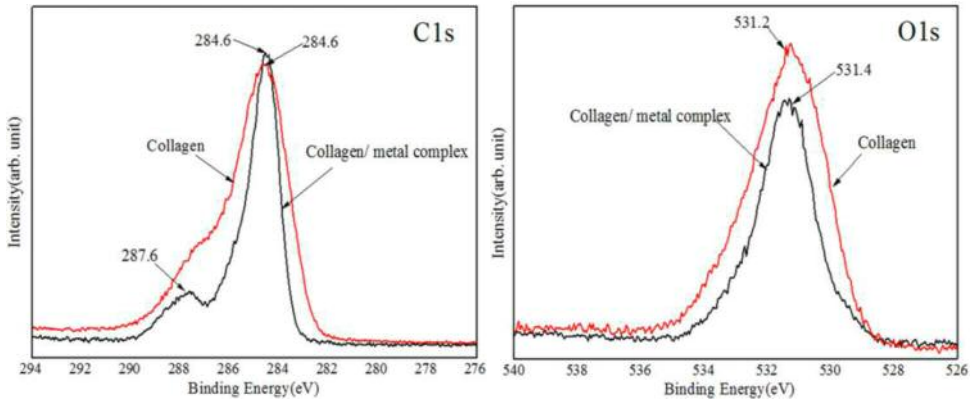


Figure 7. High resolution XPS spectra (a: collagen; b: collagen/metal complex).

after treatment is relatively greater than that of O1s, which might illustrate that there is higher possibility for N to participate in the reaction rather than O.

Table I shows that each binding energy referring to different elements has increased. Not only O and N in -COOH and -NH_2, but also that of -C-OH, N-C=O, R-C=NH. This might indicate that the -NH_2 of lysine and hydroxylysine, -NH of glutamic acid, -NH-C=NH of histidine, -CH-OH of arginine, hydroxyproline, serine and threonine, as well as the phenolic hydroxyl groups of tyrosine were all involved in interacting. Even -N-C=O of the collagen backbone.

TABLE I
XPS peaks binding energy assignments (n=3)

Element	Functional groups	Binding energy /eV Collagen	Collagen-Zr-Al-Ti
C1s	C-H, C-C	284.40 ± 0.05	284.44 ± 0.03
	C-N	285.25 ± 0.04	285.41 ± 0.03
	C-O (C-OH)	286.20 ± 0.05	286.27 ± 0.04
	C=O	287.78 ± 0.03	288.01 ± 0.03
	N-C=O	288.21 ± 0.05	288.37 ± 0.02
O1s	C=O	530.87 ± 0.04	531.20 ± 0.05
	C=O, O-C=O	531.88 ± 0.02	531.90 ± 0.01
N1s	R-C=NH, C-NH-C	399.15 ± 0.02	399.40 ± 0.01
	-NH_2 (-NH)	400.07 ± 0.01	400.13 ± 0.03

3.5 Thermal stability

The interactions between collagen and metal complex have been investigated. As is shown in Figure 8, the major peak in the DSC curve, usually used to mark the denaturation temperature of collagen, shifted from 66.51°C to 88.1°C. There are distinct differences in TG and DTG curves of collagen before and after treatment. Without any treatment, these two stages appeared at 70.9°C and 333.5°C respectively. Through interacting with Zr-Al-Ti complex, the two steps of weight loss were changed into three phases, at 72°C, 358.9°C and 595.4°C respectively. Notably, the third phase of weight loss is concerned with strong chemical bonding between collagen and the Zr-Al-Ti complex.

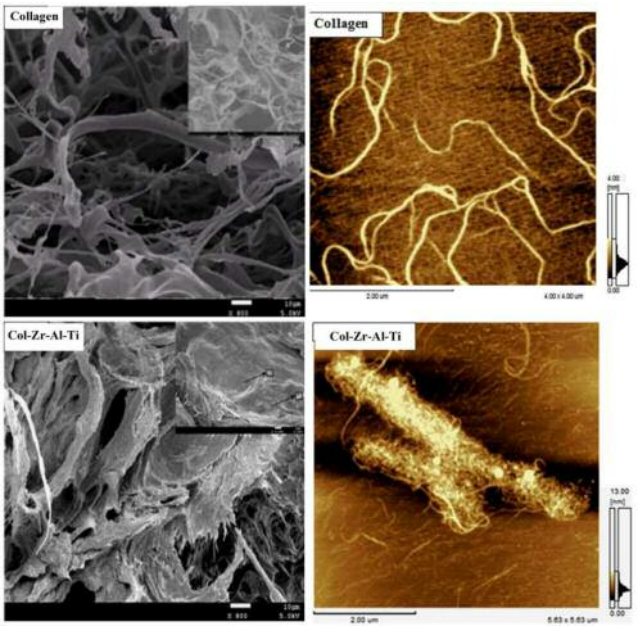


Figure 9. Morphology of collagen and collagen/ metal complex observed by SEM and AFM.

3.6 Morphology

The morphology of collagen as measured by SEM and AFM is shown in Figure 9. The introduction of Zr-Al-Ti complex has changed the collagen greatly. Before any treatment, collagen fibres are smooth and show three-dimensional structured networking. Due to the strong interactions between collagen and metal complex, the original collagen bundles held tightly to form a layer by layer structure. It is evident that Zr-Al-Ti complex has filled in collagen fibres as well as interacted with collagen's functional groups. In addition, the collagen fibres intertwined together to turn into larger size bundles, which is obvious in the AFM insert. Metal complex particles were also observed after rinsing thoroughly.

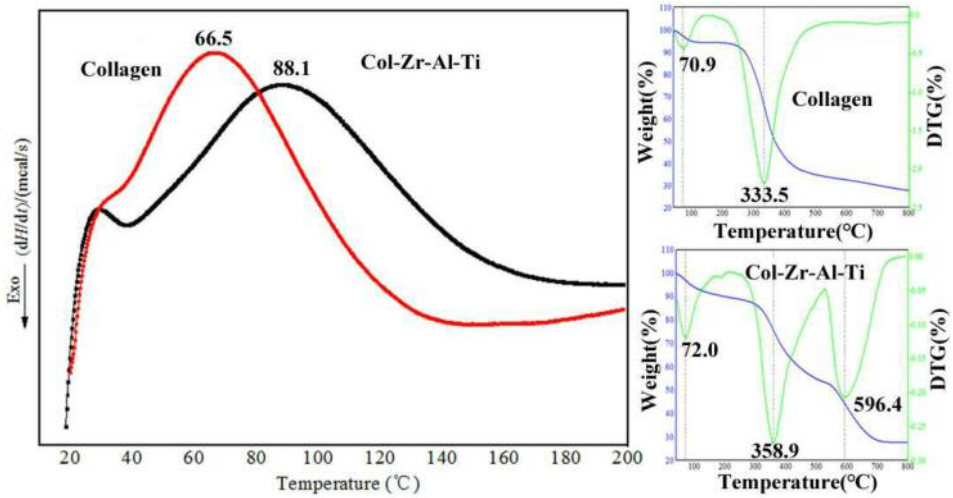


Figure 8. DSC, TG and DTG curves of collagen and collagen/metal complex.

4 CONCLUSIONS

The present study has offered a novel metal complex reacting with collagen to probe the interaction mechanism between them through computational modeling and experimental analysis. Apparently hydrogen bonds and Van der Waals' forces were observed in computational modeling. The results showed that interactions between collagen and Zr-Al-Ti complex mainly took place at the atoms N and O, which was consistent with computational simulation. A great number of amino residues were involved, especially those with $-NH_2$ and -C=O. The lone pair electrons of N and O were donors to form stable coordination bonds with central metal ions. Both physical filling and chemical crosslinking existed in collagen, leading to the increase of denaturation temperature and another phase of decomposition found from DSC and DTG analysis. However, apart from being employed in the leather industry, it still offers many opportunities and challenges for a Zr-Al-Ti complex to be applied in various fields of protein-based materials. Accordingly, numerous deep researches are essential for future development.

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