

REVIEW

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# Factors affecting thermal stability of collagen from the aspects of extraction, processing and modification

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## Abstract

Collagen, as a thermal-sensitive protein, is the most abundant structural protein in animals. Native collagen has been widely applied in various fields due to its specific physicochemical and biological properties. The beneficial properties would disappear with the collapse of the unique triple helical structure during heating. Understanding thermal stability of collagen is of great significance for practical applications. Previous studies have shown the thermal stability would be affected by the different sources, extraction methods, solvent systems in vitro and modified methods. Accordingly, the factors affecting thermal stability of collagen are discussed in detail in this review.

**Keywords:** Collagen, Thermal stability, Extraction methods, Solvent systems, Modification methods

## 1 Introduction

Leather manufactured from animal skins is extensively used for industrial commodity. The quality of leather is established on the stability of collagen which is an important structural protein in animal skins and accounts for 80–85% in dermal proteins [1]. Collagen as the most abundant protein in many tissues is widely presented not only in the skins, bones, tendons, ligaments, cartilage and blood vessels of vertebrates but also in the corneum of invertebrates and some simple multicellular organisms [2–4]. Today, 29 members of collagen have been found and could be classified as fibril-forming collagens (I, II, III, V, XI, XXIV and XXVII) and non-fibril-forming collagens [5].

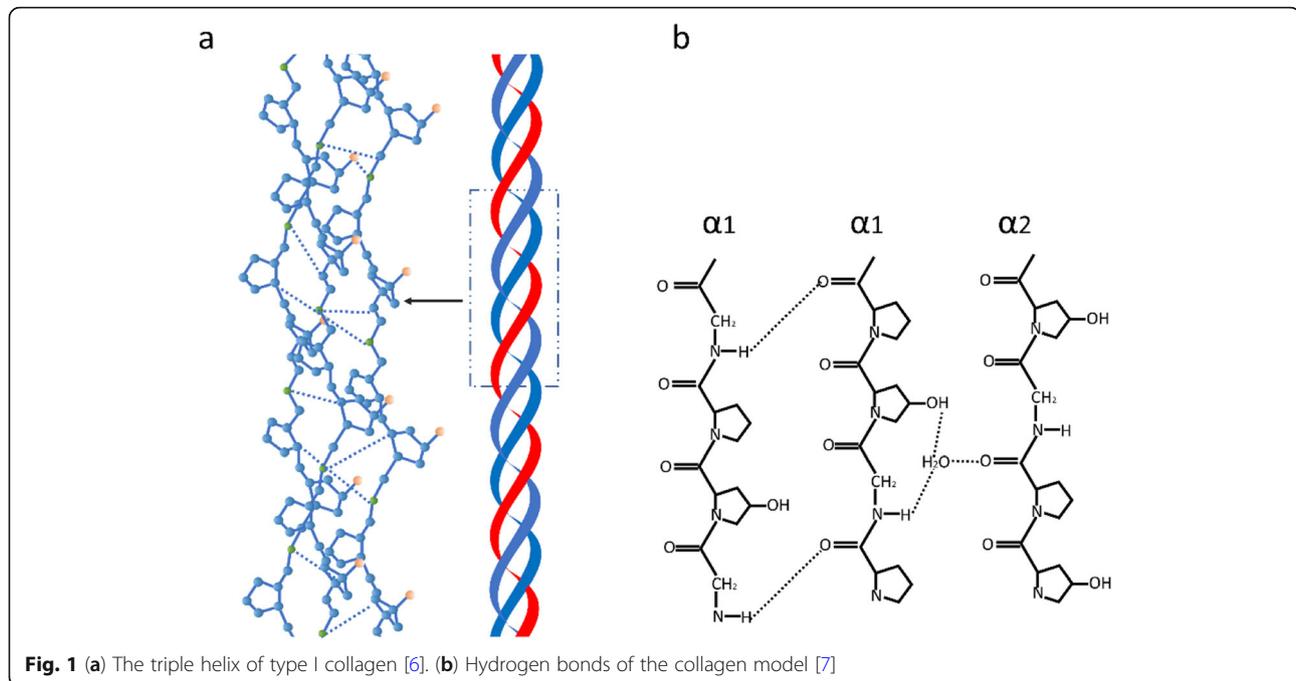
The feature of collagen is defined as containing at least one helix domain, namely triple helix. Three parallel left-hand polypeptide chains ( $\alpha$  chains) with polyproline II-type (PPII) conformation coil about each other to form a right-handed triple helix (Fig. 1a). Collagen could be a homotrimer of three identical  $\alpha$  chains as well as a

heterotrimer of two or three different  $\alpha$  chains. The amino acid sequence shows every third residue is glycine (Gly), which results in a Gly-X-Y repeating sequence where X is often proline (Pro) and Y is often hydroxyproline (Hyp) [6]. Type I collagen is the most major and abundant member in the collagen family. At present, the knowledge of collagen mostly comes from the studies of type I collagen [4]. As a heterotrimer of two different  $\alpha$  chains ( $[\alpha_1(I)]_2\alpha_2(I)$ ), Type I collagen contains 96% triple-helical domain and less non-helical domain, as shown in Fig. 2b. Non-helical domain with loose folded conformation is attributed to the absence of the Gly-X-Y repeating consequence in the N- and C-telopeptides [9, 10]. The hydrogen bonds, including N–H (Gly)  $\cdots$  O=C (X) hydrogen bond, O–H (Hyp in Y)  $\cdots$  O=C (peptide backbone) hydrogen bond and water mediated hydrogen bond, among  $\alpha$  chains stabilize collagen triple helix (Fig. 1b) [7]. Other intermolecular non-covalent bonds such as van der Waals forces, electrostatic attractions and hydrophobic bonds also play an important role in stabilizing collagen [8]. In vivo, the biosynthetic route of type I collagen is shown in Fig. 2. N- and C-propeptides of type I procollagen are cleaved by corresponding

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proteases to transform into tropocollagen. The tropocollagens laterally and longitudinally aggregate to microfibrils by self-assembly. The microfibrils are further cross-linked and eventually form macroscopic fibers and network which could be observed in tissues. As weaving into a three-dimensional architecture, the collagen fibers contribute a desirable mechanical function to skin [11].

Triple helix establishes excellent characteristics for collagen, such as fiber-forming property, biocompatibility and bioactivity, which make collagen widely used in leather industry, tissue engineering [12], pharmaceuticals [13], foods [14] and cosmetics [15]. Native collagen has been molded into different forms to adequate to the specific demands: fibers for skin in leather industry, members for sausage casing in foods, aqueous solution for a standard raw material in cosmetic formulations and gels, pellets, nanoparticles, scaffolds, sponges for biomedicines [16].

Collagen is premised on maintaining the stability of the triple helix for all biological applications. The hydrogen bonds would be easily destroyed after absorbing excessive heat, which lead to the conformation transition of collagen. The thermal denaturation would take place above a certain temperature. In general, the thermal stability of collagen is expressed by denaturation temperature ( $T_d$ ) as well as shrinkage temperature ( $T_s$ ). The higher  $T_d$  or  $T_s$ , the better thermal stability [17]. Comparing with native collagen, the thermal denatured products of native collagen (gelatin and collagen hydrolysate) exhibit quite another properties such as relative lower molecular weight, absence of conformation translation, loss of ability of fibril formation, faintly promoting effect on the adsorption and

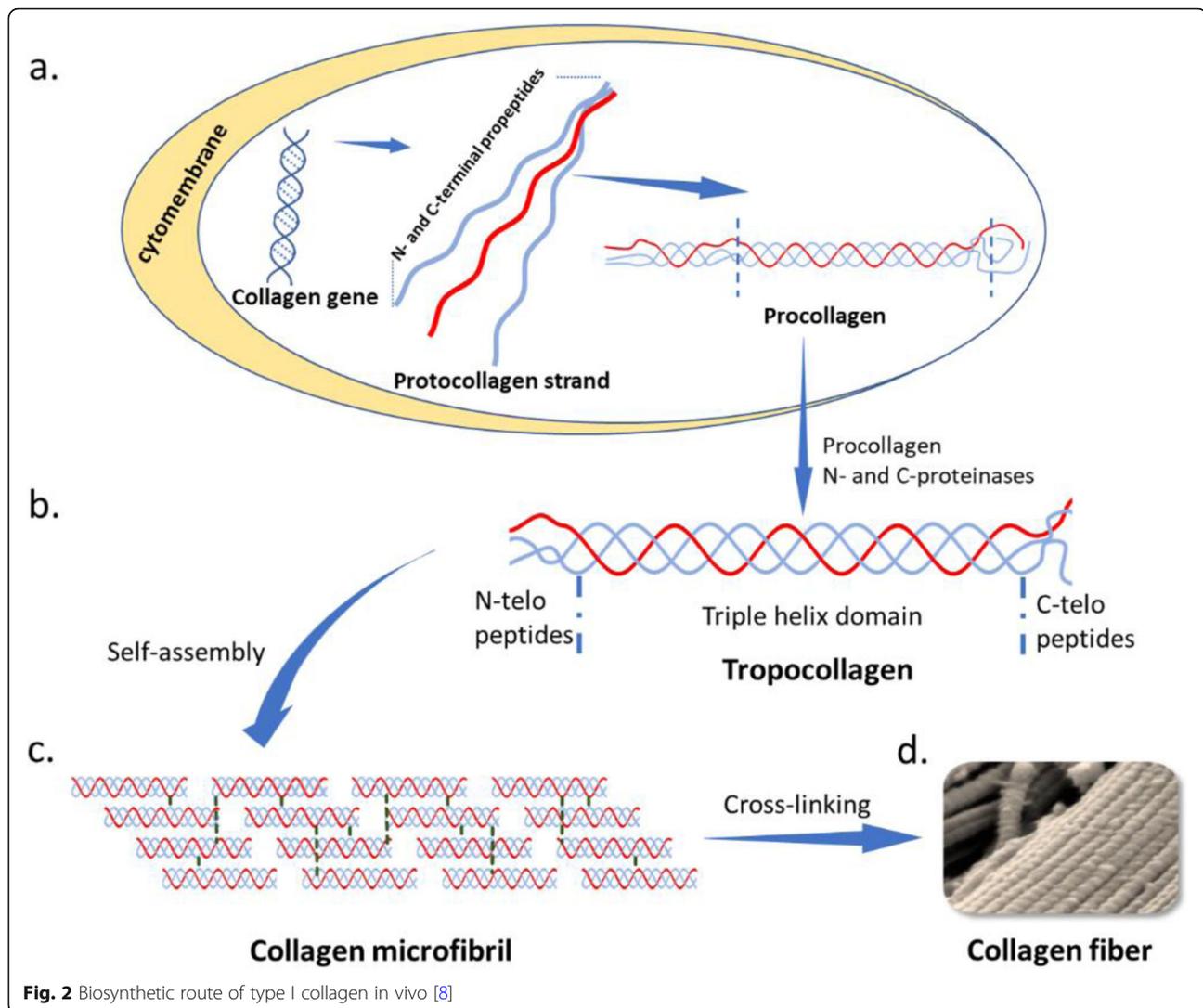
growth of keratinized cells [15, 18]. The occurrence of thermal denaturation should be prevented during processing, storage and application. The thermal stability of collagen has always been an issue of concern. This review mainly discusses the effects of extraction methods, sources, solvent systems and modification methods on thermal stability of type I collagen.

## 2 The effect of extraction methods on thermal stability

As an insoluble macromolecular structure in vivo, collagen has usually been extracted into solution in order to be further used. Native collagen with intact triple helix would be obtained by the following brief processes: pre-treating for removing the non-collagenous substances, extracting and purifying at a low temperature. The effect of the extraction methods which are commonly used or are new on thermal stability of collagen are summarized in this review.

### 2.1 Acid extraction and enzyme extraction

Acid and enzyme have been widely applied to the extraction of native collagen. Dilute acid could destroy the intermolecular salt bonds and Schiff bases, enhancing the repulsive charges on the triple helix to swell collagen fibers. Collagen extracted in acid solution still retains triple helix with amino telopeptide. Acetic acid, citric acid, lactic acid and hydrochloric acid could be used to extract collagen from tissues. Acetic acid is the best ideal acid solvent due to the high yield of extraction and bacteriostatic action [19, 20]. Acid-soluble collagen (ASC) is



commonly extracted using 0.5M acetic acid solution. Enzyme could break the highly crosslinked bonds that cannot be broken by dilute acid in the telopeptide non-helical domains of collagen to obtain the atelocollagen. Pepsin-soluble collagen (PSC), which is generally obtained using 0.5M acetic acid solution containing pepsin, has intact triple helix and shows low antigenicity thanks to the removal of telopeptides [21, 22]. The comparison of physicochemical properties between ASC and PSC has been studied by many researchers. The difference in denaturation temperature ( $\Delta T_d$ ) between ASC and PSC was more than 1°C in some literatures, as listed in Table 1. Similar thermal stability shown between ASC and PSC has also been reported: ASC (35.2°C) and PSC (34.5°C) of Nile tilapia skin [30], ASC (41.58°C) and PSC (41.01°C) of Spotted golden goatfish scale [31], ASC (32.1°C) and PSC (31.6°C) of Largefin longbarbel catfish skin [32], ASC (16.1°C) and PSC (15.7°C) of Deep-Sea Redfish skin [33],

ASC (31.5°C) and PSC (31.5°C) of Frog skin [34], ASC (26.6°C) and PSC (26.7°C) of Flatfish skin [35], ASC (34.23°C) and PSC (34.37°C) of Blacktip shark skin [36], ASC (39.3°C) and PSC (39.6°C) of Striped catfish skin [37].

The effect of those two methods on the thermal stability would be distinct owing to the different collagen sources, sample states and determination methods, as reported by Portier et al. [26] and Kozłowska et al. [38].  $\Delta T_d$  between ASC and PSC extracted from Black drum skin was 1.6°C, whilst  $\Delta T_d$  between ASC and PSC of Sheepshead skin was merely 0.3°C [26].  $T_d$  of ASC was 1.5°C higher than that of PSC determined by viscosity measurement but was 4.7°C lower than that of PSC in the form of film determined by differential scanning calorimetry (DSC) [38]. However, the intra- and intermolecular crosslinks in ASC-s was evidently richer than that in PSC-s. Additionally, ASC-s after the removal of telopeptides using the specific enzyme exhibited a

**Table 1** The difference in denaturation temperature/ $\Delta T_d > 1^\circ\text{C}$  between ASC and PSC

Sources of collagen	Td ( $^\circ\text{C}$ )		$\Delta T_d$ ( $^\circ\text{C}$ )	Determination methods of Td.	Reference
	ASC	PSC			
Skin wastes of marine eel fish <sup>c</sup>	39	35	4 <sup>a</sup>	Viscometer	[23]
Skulls of skipjack tuna <sup>c</sup>	17.8	16.6	1.2 <sup>a</sup>	Viscometer	[24]
Spines of skipjack tuna <sup>c</sup>	17.6	16.5	1.1 <sup>a</sup>	Viscometer	[24]
Bone of Spanish mackerel <sup>c</sup>	18.02	16.85	1.17 <sup>a</sup>	Viscometer	[25]
Skin of black drum <sup>c</sup>	34.2	35.8	-1.6 <sup>b</sup>	Viscometer	[26]
Loach skin <sup>c</sup>	36.03	33.61	2.42 <sup>a</sup>	CD	[27]
Ovine bones <sup>c</sup>	42.31	38.91	3.4 <sup>a</sup>	DSC	[28]
Skin of grass carp <sup>d</sup>	69.04	62.2	6.84 <sup>a</sup>	DSC	[29]

CD Circular dichroism measurements, DSC Differential scanning calorimetry

<sup>a</sup>Td of ASC is higher than that of PSC

<sup>b</sup>Td of PSC is higher than that of ASC

<sup>c</sup>Collagen solution

<sup>d</sup>Lyophilized collagen

relative lower rate of fiber formation and a weaker collagen gel strength [39–41]. The telopeptides rich in hydrophobic residues are helpful for inter-microfibrillar interaction [42]. Accordingly, telopeptides might make a contribution to the thermal stability of collagen after fiber formation.

## 2.2 Alkali extraction

Back in early 20th century, several researchers investigated the effect of mild alkaline treatment on collagen, because liming is an essential process in the conversion of skins to leathers. Bowes et al. [43] suggested the hydrolysis of amide groups was the main reaction with a little breakage of some peptides when insoluble collagen was treated with alkali at pH 13.0. Courts [44] further proved the progressive hydrolysis of labile peptide bonds occurred in alkaline pretreatment of insoluble collagen. People focused on remaining collagen insoluble during alkaline treatment by keeping the pH value below 13 or using swelling restrainers [45]. Little work was described about the fraction soluble in alkali until 1971 by Kemp et al. [46]. A fraction of high molecular weight was isolated from cow bone ossein with 5% (w/v) NaOH and 0.1 M NaCl. The resulting material merited the description of 'collagen' owing to high content of the characteristic helix observed by optical-rotation studies. The Td values of the alkaline-soluble collagen was about 4 $^\circ\text{C}$  lower than that of ASC extracted from cow skin [46]. Type I collagen was successfully extracted from bovine skin by Hattori et al. [47] using 3% hydroxide and 1.9% monomethylamine at 4 $^\circ\text{C}$  for 1d. The thermal stability of the alkaline-soluble collagen (38.0 $^\circ\text{C}$ ) was lower than that of ASC (40.4 $^\circ\text{C}$ ). Similar results were reported by others [48, 49]. Td of the alkaline-soluble collagen was 5 $^\circ\text{C}$  lower than that of ASC-s and PSC-s extracted from shark skin [48]. The thermal stability of the alkaline-

soluble collagen was lower than that of PSC extracted from pig skin and the Td values were 34.5 and 37 $^\circ\text{C}$  respectively [49].

Alkali dissolved collagen through removing the telopeptides involved in crosslinkage of the molecules and could lead to the deamination of Asn and Gln into Asp and Glu. For the loss of telopeptides and the generation of the stronger electrostatic repulsion of Asp and Glu, the native collagen extracted with alkali has slightly less thermal stability comparing with ASC-s and PSC-s.

## 2.3 Ultrasonic assisted and microwave assisted extraction

Ultrasonic irradiation, as a safe, reliable, reproducible and environmentally friendly technology, is widely used to demonstrate physicochemical properties in low-intensity and promote emulsification, cell destruction and chemical reactions in high-intensity [50]. Cavitation as the most usual property of ultrasonic produces intense physical forces to provide sufficient energy for an extraction process [51]. Akram et al. [52] extracted acid-soluble type II collagen from chicken sternal cartilage using ultrasonic assist (950W, 20–25kHz, amplitude

10 for 24min). Td of the collagen was 5 $^\circ\text{C}$  higher than that of the un-ultrasonic treated collagen. Qu et al. [53] extracted pepsin-soluble type I collagen from bovine hide via ultrasonic assist in the optimal condition (20kHz and 90W for 20min). Td of the collagen (40.09 $^\circ\text{C}$ ) was similar to that of PSC (39.87 $^\circ\text{C}$ ). With the help of ultrasonic, native collagen has been successfully obtained in a short time with high yield [54–57]. It should be noted that an evident degradation of collagen would take place after ultrasonic treatment for a longer duration time or under a higher degree of amplitude, as reported by Kim HK et al. [58, 59]. The effects of different ultrasonic amplitudes and duration time on ASC extracted from sea bass (*Lateolabrax japonicus*) skin in

control of the same temperature and frequency (4°C, 20kHz) were investigated. The collagens treated below 60% amplitude for 6h still exhibited a typical type I collagen structure, including  $\alpha 1$ ,  $\alpha 2$  and  $\beta$  chains, which were observed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns. Gradual degradation occurred as the ultrasonic time lengthened and the amplitude increased, which was observed as the rapid increase of the components and the wide distribution of molecular weight [58]. Excessive physical forces generated from cavitation might break the hydrogen bonds among collagen molecules and weaken van der Waals interaction among the polypeptide chains, leading to collagen denaturation [59].

Microwave irradiation as heating energy source or transmission medium is still a new technology to be applied to the extraction of native collagen [60]. Li et al. [61] isolated collagen with papain from pigskin by microwave pretreatment at 25°C for 2 min. The yield of collagen observably increased from 51.24% to 76.72%. The collagen with intact structure was extracted from cattle hide by Cheng et al. [62] using microwave irradiation at 37°C for 7 h as the ratio of material to liquid (0.5 mol/ml acetic acid) was 1:45 (w/v). Td of the collagen treated with microwave irradiation was 38.8°C, which was similar to that of the collagen extracted under water bath heating (38.91°C). The yield of collagen extracted by microwave irradiation was 1.5 times higher than that of traditional water bath heating. The thermal denaturation behavior of the bovine tendon collagen in acetic acid solution (5.0 mg/ml) during microwave irradiation and oil-bath heating was examined by combination of CD and polarimetry [63]. The feature of native collagen is revealed in CD spectra at positive peak around 210–230 nm and at negative peak around 190–200nm [64]. The positive peak (223nm) of collagen treated microwave irradiation disappeared at 40°C and that of oil-bath heating disappeared at 50°C. The significant change of relative optical rotation variable of collagen under microwave irradiation started at 25°C, which was 9°C lower than that of oil-bath heating (34°C). The thermal denaturation of collagen in acetic acid solution was more easily accelerated under microwave irradiation than under conventional heating. When being applied to extraction of collagen with a lower denaturation temperature, the microwave irradiation should be used with caution.

#### 2.4 Ionic liquids pretreatment extraction

Owing to the tight weave of collagen in tissues, some chemical reagents have been used to remove non-collagenous constituents and to loosen fiber contexture for extracting collagen easily [65, 66]. Instead of traditional reagents, Ionic liquids (ILs) composed of bulky organic cations and small inorganic anions have been

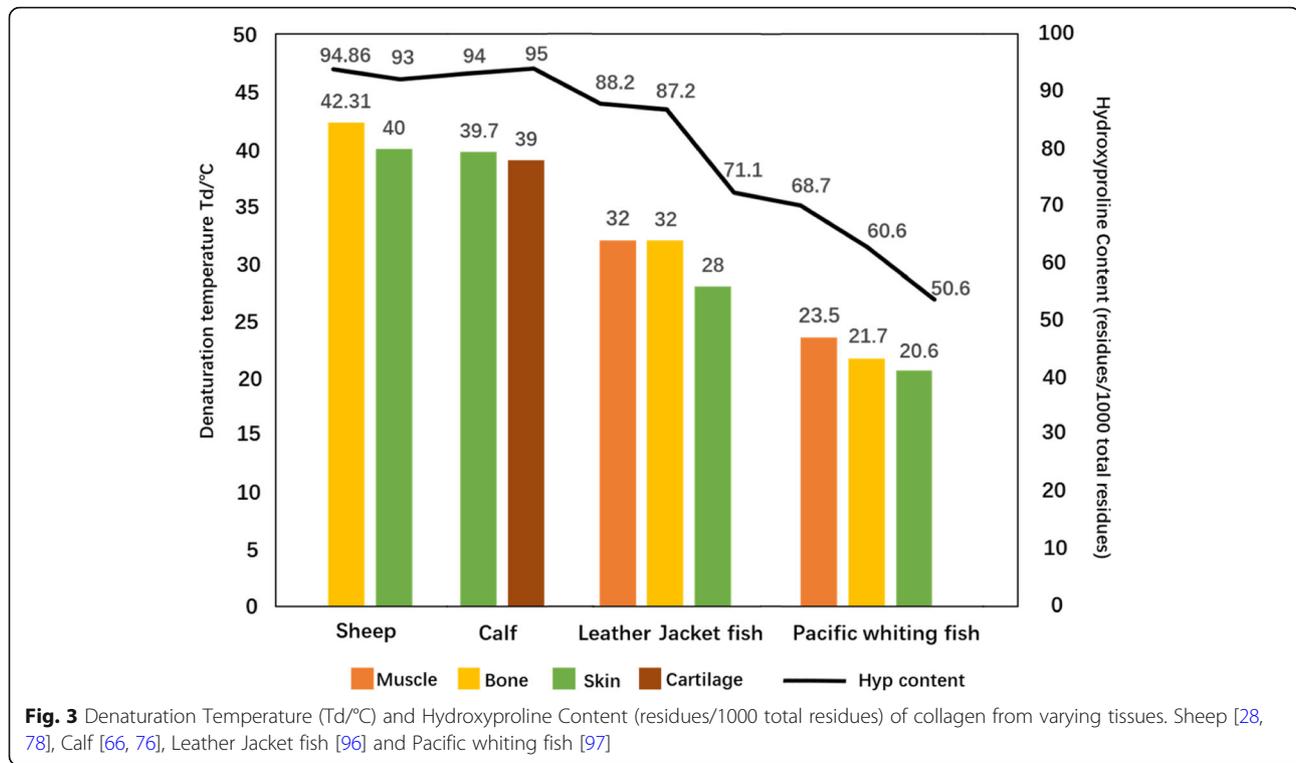
applied to the pretreatment of skin [67]. Choline thioglycolate (LD50-Oral-rat: 3400 mg/kg) rather than sodium sulphide (LD50-Oral-rat: 246 mg/kg) was used to remove hair from goat skin. The tensile strength and tear strength of the skin treated with choline thioglycolate were better than that of sodium sulphide, which were 22.7 MPa, 56.4 N/mm and 15.8 MPa, 40.1 N/mm respectively [68]. 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) as a fiber opening reagent was used to treat wet salted goat skin. The T<sub>s</sub> values of the pelts treated with 0.25–1.0% [BMIM][Cl] (108–116°C) were similar to that of conventional process (112°C) [69]. 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF<sub>4</sub>]) was employed in both the unhairing and fiber-opening processes of wet salted goat skin. The T<sub>s</sub> values of the leather treated with [BMIM][BF<sub>4</sub>] and that treated with calcium hydroxide and sodium sulfide were 111 and 116 °C respectively [70]. ILs could help to open collagen fibers and take little damage to the structure of collagen, which make ILs feasible to assist in extracting native collagen. Liu et al. [71] firstly used two ILs, namely 1-ethyl-methylimidazolium dicyanamide ([EMIM] [N (CN)<sub>2</sub>]) and 1-ethylmethylimidazolium tetrafluoroborate ([EMIM][BF<sub>4</sub>]), of different concentrations (30, 50 and 70%) to pretreat calfskin at 4°C for 24h as the ILs/skin ratio was 10:1(w/w). Then PSC-s were extracted from the skins pretreated with the ILs, which were observed to always consist of  $\alpha 1$ ,  $\alpha 2$  and  $\beta$  chains in SDS-PAGE patterns. The extraction yield of collagen increased by about 9% after pretreating skins with the 70% ILs. The Td values of collagen decreased less than 1°C as the concentration of the ILs increased from 0 to 70%. ILs might not destroy the triple helix but affect interaction among collagen molecules at low temperature.

The native collagen with distinct properties would be obtained by different extraction methods. Assistant means could help to raise the extraction yield but would destroy the triple helical structure of collagen under an inappropriate condition. The extraction methods and assistant means could be adjusted according to practical conditions and applications.

### 3 The effect of collagen extracted from different sources on thermal stability

In the collagen family, the fibril-forming collagens, especially type I–III collagens, have been the most widely used because of the relatively abundant sources. Type I collagen is the major component in skin, bone, tendon, ligament and cornea. Type II collagen mainly exists in cartilage, vitreous body and nucleus pulposus. Type III collagen which often co-distributes with type I collagen resides in the skin, blood vessels and intestines [72]. The distribution of collagen members in organisms is quite different. The choice of source rich in desired collagen is of importance. Skin of porcine, bovine and sheep with





( $T_d = 69.9 - 3105 / N_{\text{Hyp}}$ ) was linearized from all the data except for that of the ice fish collagens. For most collagens, the thermal stability is in the positive correlation with the content of Hyp. A few collagens of invertebrate deviate considerably from the trend. The collagen of *Lumbricus terrestris* (earthworm) had lower  $T_d$  (22°C) but higher amount of Hyp (~17%) [106]. The collagen of *Riftia pachyptila* cuticle exhibited higher  $T_d$  (37°C) but contained lower levels of imino acids (~5%). 18% Thr rather than Hyp was found in the Y position by analyzing the amino acid sequences of the collagen isolated from *Riftia pachyptila* cuticle. Thr was proposed to replace Hyp in the triple helix for stabilizing function [107]. The effect of the individual amino acid and various amino acid sequences on stability of collagen is difficult to achieve by direct determination of native collagen with complex structure. Crystallization of native collagen and collagen-like domains is necessary. Collagen-like peptides have been crystallized to further confirm the basic triple helix model and elucidate the hydrogen-bonding patterns in detail [108–111].

The relationship between amino acids and the thermal stability of collagen was evaluated via a “host-guest” peptide which had the following sequence  $-(\text{Gly-Pro-Hyp})_3\text{-Gly-X-Y-(Gly-Pro-Hyp)}_4-$ . The Gly-Pro-Hyp repeating sequence in both ends provided a stability triple-helical environment for the guest Gly-X-Y (GXY) triplet. All of the host-guest peptides had characteristic of the collagen triple helix in CD spectrum. The melting temperature

( $T_m$ ) of all different GXY was determined by CD [112, 113]. Initially, the most frequent 5 non-polar residues in collagens were evaluated by host-guest studies. The peptide where GXY = Gly-Pro-Hyp was the most stable ( $T_d = 44.5^\circ\text{C}$ ). The replacement of a single Pro or Hyp dropped the thermal stability (20.7°C–39.9°C) [112]. All 20 possible amino acids in the X and Y positions were further studied. (Gly-Pro-Hyp)<sub>8</sub> had the highest  $T_m$  value of 47.3°C. The change of identity in the X position showed a small effect on thermal stability (31.9°C–47.3°C). The replacing of Hyp in the Y position led to large spread of thermal stability (26.0°C–47.3°C). Arg in the Y position like Hyp is also one of the most stable residues, because Arg is involved in hydrogen bonds and hydrophobic interactions [113]. The importance of imino acids was confirmed. The effect of Hyp in the Y position on stabilization was greater than that of Pro in the X position.

For vertebrate collagen, Hyp as the form of 4R-hydroxy-L-proline (4R-Hyp) is found exclusively in the Y position to confer extra stability [114, 115]. Two controversial mechanisms of Hyp stabilizing the triple helix were explained by crystallographic evidences. One mechanism was pointed out by Brodsky and her colleagues [116, 117], that was Hyp stabilized triple helical structure by water mediated hydrogen bonds. Water bridge molecules linked the hydroxyl group of Hyp residues to a carbonyl group on the backbone of an adjacent chain via hydrogen bonds. The triple helical structure of  $\text{NH}_2\text{-Gly-(Gly-Pro-Hyp)}_4\text{-Gly-Pro-Hyp-(Gly-Pro-Hyp)}_3\text{-GlyAc}$  was less stable than that of  $\text{NH}_2\text{Gly-(Gly-Pro-Hyp)}_8-$

GlyAc. The replacement of Hyp with Flp was suggested to disrupt the hydration induced by Hyp, because Flp could not form strong hydrogen bonds [118]. The other mechanism was proposed by Raines et al. [119, 120]. Td of (Gly-Pro-Flp)<sub>10</sub> was much higher than that of (Gly-Pro-Hyp)<sub>10</sub>, which were 91 and 69°C respectively. Because Flp is more electronegative comparing with Hyp, the stereo electronic inductive effect rather than water bridges was suggested to play a major role in triple helix. Hyp was proposed to stabilize the trans conformation of the imide peptide bond through stereoelectronic inductive effect, because all peptide bonds are trans. In some invertebrate and basement membranes collagens, Hyp occurred in both X and Y positions [121, 122]. Hyp in the X position was demonstrated to exhibit effects of stabilizing or destabilizing on triple helix. Td of (Gly-Hyp-Hyp)<sub>10</sub> was 4°C higher than that of (Gly-Pro-Hyp)<sub>10</sub>. Hyp-induced stabilization in the X position attributed to the interchain dipole-dipole interactions between proximal hydroxy bonds of adjacent Hyp residues [123, 124]. Comparing with (Gly-Pro-Pro)<sub>10</sub>, the failure of (Gly-Hyp-Pro)<sub>10</sub> to form a triple helical structure demonstrated Hyp in the X position could destabilize the triple helix [120, 125]. Since Hyp preferred the exo ring pucker, Hyp was proposed to be improper for the X position where a Pro residue adopted the endo ring pucker to stabilize a triple helix [125, 126]. Hyp-induced stabilization in the X position is supposed to occur only when the residue in the Y position is able to provide extra-stabilizing interactions. Hyp-induced stabilization in the Y position is independent of the residue type in the X position [127].

Thr replaced Hyp in the Y position to stabilize the triple helix in the cuticle collagen of *vestimentiferan Riftia pachyptila*. The triple-helix structure was demonstrated to achieve only after glycosylation by analyzing a scale of synthetic Gly-Pro-Thr peptides [128, 129]. Glycosylated threonine was speculated to stabilize the triple helix in a way similar to proline ring pucker, inductive effects or hydrogen bonding [130].

Imino acids as a major role in most collagens make a contribution to thermal stability. Other mechanisms of stabilization, such as Arg in the Y position and glycosylation of Thr, might work in a collagen of lacking imino acids. The effects of amino acid compositions on the thermal stability of collagen mainly came from the studies of idealized sequences. Whereas, amino acid sequences are much more complex in native collagens than that in ideal model peptides. Collagen of various sources shows a different expression of molecules to satisfy the biological function.

For the first time, collagen model fragments with natural amino acid sequences were constructed by Li and her group [131] using computer simulation. To further

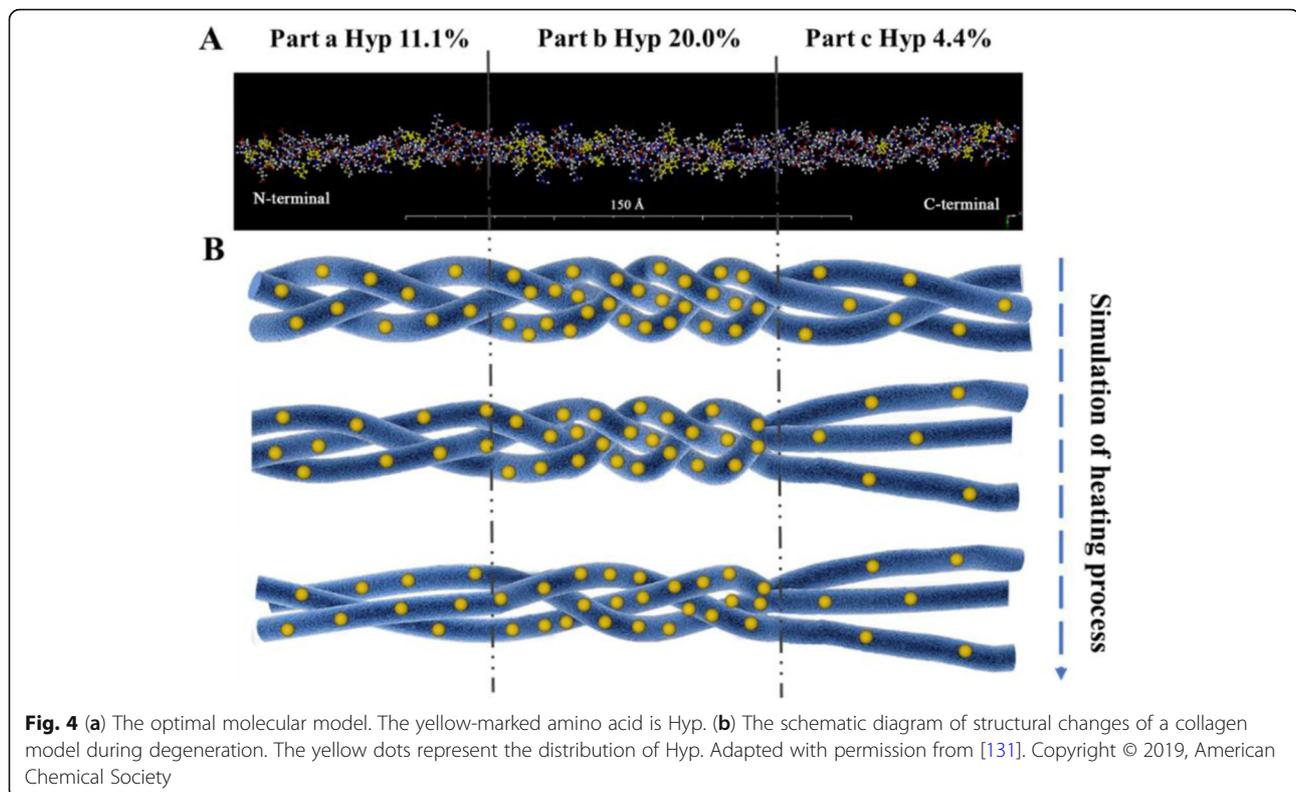
study the effect of Hyp content on thermal stability of collagen molecules, the fragments of grass carp collagen were selected according to the distribution of Hyp content in diverse regions of amino acid sequences. The model fragments were optimized to fit the typical triple helix. A mathematical model was carried out to estimate the hydrogen bond energy of intact collagen chains according to the model fragments. The hydrogen bond energy between the collagen chains (17.74 J/g) calculated by the mathematical model was consistent with the DSC results (17.98 J/g). The lowest energy reduction (1.247 kcal/mol) was shown in the simulated fragment of the highest Hyp content (15.6%) after heating simulation at 35°C. The collapse of the triple helix began in the regions of less Hyp content and then in the regions of high Hyp content, as shown in the simulation of structural change of model fragments during heating process (Fig. 4). The physical and chemical properties of native collagen could be further explored by constructing collagen model fragments with natural amino acid sequences.

#### 4 The effect of different solvent systems on thermal stability

Collagen is always used in form of solution or is developed with aqueous preparations. The triple helix of collagen is maintained by several interactions such as hydrogen bonds, electrostatic attractions and hydrophobic interactions. These interactions might be disturbed by the change of solvent systems, thus affecting the thermal stability of collagen. This review mainly discusses the thermal stability of collagen in different solvent systems from four aspects.

##### 4.1 The concentration of collagen

Td of the collagen extracted from bovine skin in acid solution was observed to decrease by about 0.8°C as the collagen concentration increased from 5 to 20 mg/ml in Liu's study, which indicated collagen molecular state might change in different collagen concentrations [132]. Three collagen concentrations were chosen according to the critical aggregation concentration (CAC) of collagen (bovine skin) in acid solution [133]. 0.25 mg/ml expressed non-aggregation state of collagen molecules. 0.5 and 1.0 mg/ml exhibited critical aggregation and complicated aggregation state respectively. Transition temperature of collagen slightly decreased by 0.3°C as collagen concentration increased from 0.25 to 1.0 mg/ml. The morphology of collagen solution changed from homogenous to inhomogeneous and the collagen fibers gradually became denser with the increase of collagen concentration. The aggregation behavior was induced by increasing collagen concentration in acid solution, which was proposed to be largely mediated through the interactions of aromatic residues. The interaction between



the aromatic residues in one monomer and imino acids residues within another monomer caused the aggregation of collagen [134]. The triple-helical conformation was proposed to be buttressed through ordered water networks between adjacent monomers [135]. In a higher collagen concentration, more hydrophobic microdomains were formed during the process of aggregation. Ordered water networks might be destroyed, which resulted in a slightly decrease of thermal stability. In the other hand, a higher rate of heat transfer occurred during heating process because of the shorter distance among collagen molecules in an aggregation state [133]. From a thermodynamic point of view, the aggregation is an entropy-driven process described as randomness reduction in the spatial arrangement of the collagen monomers in solution [136]. The thermal denaturation behavior of collagen could also be described as a thermodynamic process [132, 136]. Lumry-Eyring model was the most probable mechanism for estimating the thermal denaturation of collagen and showed a process of gradual unfolding of triple-helical structure during heating. Collagen existed three states, namely native triple helix state, the partially unfolded state and the denatured state, during thermal denaturation. The quantity of those three states would be changed with the varying temperature and heating rates [137].

#### 4.2 The concentration of acetic acid

Acetic acid (AA) is regarded as the most ideal solvent in extracting and dissolving collagen. Collagen could be efficiently extracted in and homogeneously dispersed into AA solution due to the electrostatic interaction or lyophilic hydration of AA. Yang et al. [138] dissolved lyophilized collagen of calf skin into 0.1–2.0 M AA solution respectively and kept collagen concentration at 0.5 mg/ml. The denaturation temperatures ( $T_{m2}$ ) of the collagen soluble in 0.1M AA solution and in 2.0M AA solution were 42.56 and 34.76°C respectively.  $T_{m2}$  was a clear negative linear correlation with the AA concentration ( $C_{AA}$ ), as described in the formula  $T_{m2} = 44.30 - 1.93 \times C_{AA}$ . The characteristic CD spectra of native collagen exhibits a positive peak around 210–230 nm and a negative peak around 190–200nm [64]. All samples exhibited the typical CD spectra of native collagen conformation. The ratio of positive to negative peaks (Rpn) could be used to indicate the integrity of triple helical structure. The Rpn values of collagen in 0.1 M and 0.5 M AA solution almost the same, which were 0.132 and 0.133 respectively. Further increasing AA concentration, Rpn first increased to 0.145 and then decreased to 0.129. Native collagen structure remained in the AA solution of different concentrations but the conformation of collagen was affected. Additionally, the morphology of

collagen in low AA concentrations (0.1–1.0 M) was observed as inhomogeneous aggregation state. Collagen morphology gradually became homogenous and the fibers get thinner as the AA concentration was above 1.0 M. Disaggregation would take place in a higher AA concentration. The repulsion of  $\text{NH}_2^+$  groups would be enhanced with the increase of free  $\text{H}^+$  concentration released by increasing AA concentration. The water mediated hydrogen bonds among collagen molecules would be gradually disrupted as AA concentration increased. Intra- and inter-molecular hydrogen bonds were weakened with the increase in AA concentration, thus leading to the decrease of collagen thermal stability.

#### 4.3 Different inorganic ion concentrations and species

In aqueous solution, ions might interact with collagen and/or water molecules to control the state of collagen. The effect of inorganic salt on protein as “salting-in” and “salting-out” have been well known. Specific ion effects were first proposed by Hofmeister [139], which was now known as the Hofmeister series. The Hofmeister series is the ordering of ions in terms of their ability to salt out or salt in proteins as follows:  $\text{SO}_4^{2-} > \text{HPO}_4^{2-} > \text{CH}_3\text{COO}^- > \text{F}^- > \text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^- > \text{ClO}_4^- > \text{SCN}^-$  and  $\text{Mg}^{2+} > \text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{NH}_4^+ > (\text{CH}_3)_4\text{N}^+$ . Generally, kosmotropes on the left of  $\text{Cl}^-$  and  $\text{K}^+$  were called “water structure makers” which make the bulk water more ordered and collagen preferentially hydrated to keep the original state of collagen. Chaotropes on the right were called “water structure breakers” which reduce the strength of intrahelical hydration and promote the denaturation of collagen via increasing the translational and vibrational frequencies of the water molecules [140]. The effect is dominated by anions which are more polarizable and strongly hydratable [141, 142]. Komsa-Penkova et al. [143] chose 12 inorganic salts to study the effect of different ion concentrations and species on thermal stability of collagen. The salts included sodium salts with  $\text{Cl}^-$ ,  $\text{SCN}^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{HPO}_4^{2-}$ ,  $\text{SO}_4^{2-}$  anions and chloride salts with  $\text{Li}^+$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{NH}_4^+$ ,  $\text{Ca}^{2+}$  cations.  $\text{Li}_2\text{SO}_4$  and  $\text{NaN}_3$  were also measured. The salts were dissolved in 50 mM acetic acid to get different concentrations. Then the salt solutions and collagen solutions (in 50 mM acetic acid) were mixed at the ratio of 1:1 (v/v) and the final collagen concentration was kept at 0.5 mg/ml. The change of the pH values of the samples, except  $\text{Na}_2\text{HPO}_4$ , between 3 and 4.5 was proposed not to affect Td. At low salt concentrations of below 20 mM, Td of the all collagen solutions decreased by about 0.2°C for every 1 mM increase in concentration. At intermediate salt concentrations of roughly 20–500 mM, the anions dominated the change of thermal stability of collagen and followed the order  $\text{H}_2\text{PO}_4^- \geq \text{SO}_4^{2-} > \text{Cl}^- > \text{SCN}^-$ . The Td values of collagen in  $\text{NaH}_2\text{PO}_4$  (20–500

mM) and in  $\text{Na}_2\text{SO}_4$  (50–100 mM) slightly fluctuated as concentration increased, which were  $37.6 \pm 0.3$  and  $35.5 \pm 0.1^\circ\text{C}$  respectively.  $\text{NaSCN}$  and all the chloride salts decreased the thermal stability of collagen and the tendency induced by the former was more obvious. Td of collagen decreased by about 6°C as the concentration of  $\text{NaSCN}$  increased to 150 mM and the same effect could be obtained in 450 mM  $\text{NaCl}$ . The increase of the Td values in  $\text{Na}_2\text{HPO}_4$  might be affected by the pH value because of the significant increase of pH from 6.0 at 10 mM up to 9.6 at 500 mM  $\text{Na}_2\text{HPO}_4$ .  $\text{SO}_4^{2-}$  anions interacted specifically with collagen. Td of collagen existed both a lower value and a higher value in  $\text{Na}_2\text{SO}_4$  (100–150 mM) and  $\text{Li}_2\text{SO}_4$  (300–750 mM) solutions, for example, the Td values were 32.2°C and 45.4°C in 150 mM  $\text{Na}_2\text{SO}_4$  and were 36.3°C and 43.7°C in 300 mM  $\text{Li}_2\text{SO}_4$ . The reason was inferred to be the presence of a complex two-step denaturation process or two fractions of dissolved and salted-out collagens. At higher concentrations (above 500 mM), a precipitous increase of thermal stability took place due to the observable decrease of free water in collagen induced by salting-out.

#### 4.4 Ionic liquids (ILs)

Ionic liquids (ILs) are commonly defined as consisting of asymmetrical bulky cation and symmetrical small anion. ILs generally display low melting points and some are liquid at room temperature due to a larger volume distribution of the charge of the cation and anion [144, 145]. Organic cations in ILs mainly include imidazolium, ammonium, phosphonium, cholinium and pyridinium and anions can be various organic or inorganic ions. ILs of different concentrations and types would show distinct effect on the thermal stability of collagen, as reported by Tarannum and her colleagues [146, 147]. Collagen of rat tail tendon (RTT) was treated with diethyl methyl ammonium methane sulfonate (AMS) of different concentrations. The concentration of collagen was kept at 2.7 μM (pH 4.0) after being mixed with AMS at collagen/AMS ratios from 1:0.05% to 1:10% (v/v). RTT fibers were treated by 0.05–10% AMS at 25°C for 24h. Td of the collagen solutions slightly decreased by 1°C with the increase of AMS. The Td values of collagen fibers gradually reduced from 63°C (0% AMS) to 55°C (5% AMS) and then obviously decreased to 48°C as AMS was 10%. AMS was shown not to affect the secondary structure of collagen and just to decrease thermal stability at inter-fibrillary level [146]. RTT collagen solution was added with the other two ILs, namely bis-choline sulphate (BCS) and 1-butyl-3-methyl imidazolium dimethyl phosphate (IDP), of same concentration (10%) respectively to make final collagen concentration of 1.33 μM (pH 4.0) at 4°C. The small pieces of collagen fiber were prepared after incubation at 25°C for 24h to further be

determined by DCS and CD. Td of the collagen treated with BCS was 5°C higher and that of the collagen treated with IDP was 17°C lower than that of the native collagen fibers. The collagen added with BCS showed the typical CD spectrum of native collagen. However, both positive peak (222 nm) and negative peak (197 nm) completely disappeared in the CD spectrum of the collagen added with IDP [147]. Mehta et al. [148] found collagen could be dramatically stabilized by choline dihydrogen (cDHP). Collagen of RTT was added with cDHP of different concentrations from 0.05 to 10% (w/v) to keep the final collagen concentration at 0.15 mg/ml (pH 4.0). The collagen added with 0.05% (w/v) genipin was considered as positive control (CG). The solutions were incubated at 4°C overnight and then air dried on polyethylene plates to form membranes. Td of the membranes were measured by microshrinkage tester. Meanwhile, the degree of crosslinking (crosslinking efficiency) of the solutions were determined using TNBS assay. The Td values of collagen treated with cDHP showed a linearly upward with the increase of cDHP concentration, which increased by 20°C as cDHP reached to 10%. Crosslinking efficiency of collagen added with 0.05% cDHP was similar to CG (about 30%). The crosslinking efficiency of collagen was remarkably increased as the concentration of cDHP above 0.05%, which reached to 89% for 10% cDHP. As a potential crosslinker, phosphate anion of cDHP was postulated to attached to cationic functional groups of collagen via electrostatic interaction to make collagen chains closer, resulting in the formation of crosslinking among collagen molecules. The aggregation behaviors of collagen molecules (calf skin) in a novel acetic acid/1-ethyl-3-methylimidazolium acetate (AA/[EMIM][Ac]) biphasic solvent was studied by Yang et al. [149]. The blends of collagen and AA/[EMIM][Ac] were kept the collagen concentration constant using the varying AA/[EMIM][Ac] ratios of 10:0 to 5:5 (v/v) where AA was 0.1 M. The denaturation temperature of collagen decreased from 42.523 to 38.138°C and the average size of collagen fibers gradually decreased from 1947.66 to 806.06 nm as AA/[EMIM][Ac] ratios increased from 10:0 to 5:5. The samples exhibited the typical SDS-PAGE patterns of native collagen, including  $\alpha 1$ ,  $\alpha 2$  and  $\beta$  chains. The hydrogen bonds formed by the amino hydroxyl and ester oxygen groups among adjacent collagen monomers were broken in the AA/[EMIM][Ac] solutions, because two stronger hydrogen bonds were formed between  $\text{Ac}^-$  and the amino hydroxyl groups and between  $\text{EMIM}^+$  and the ester oxygen groups. The thermal stability of collagen solution was weakened as [EMIM][Ac] increased.

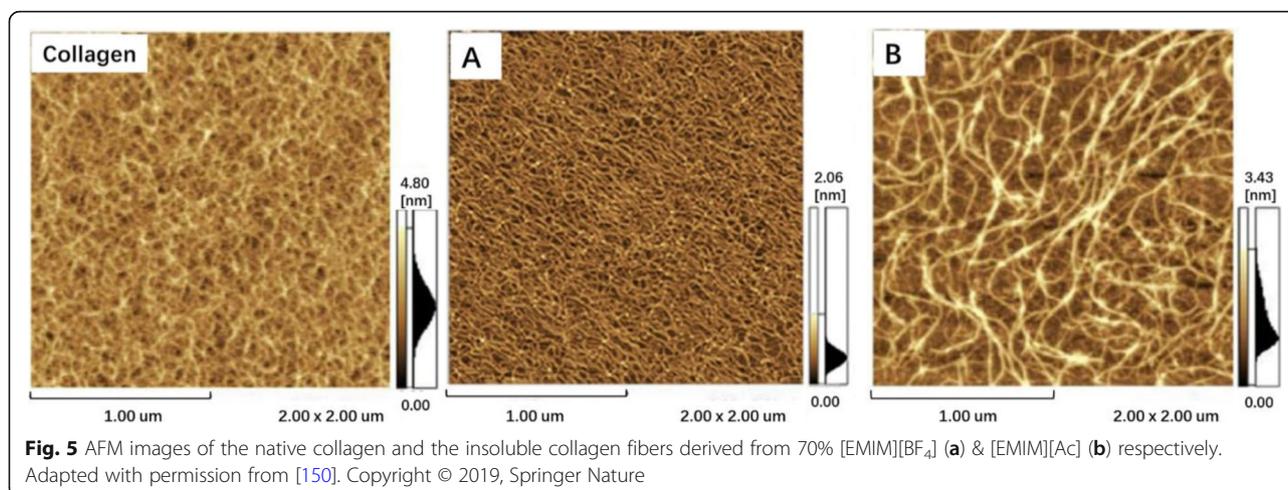
The dissolution and thermal stability of collagen in ILs of different polarity and concentrations were investigated by Liu et al. [150]. Two EMIM-based ILs with anions of

tetrafluoroborate ( $\text{BF}_4$ ) and acetate (Ac) were chosen. [EMIM][ $\text{BF}_4$ ] had low polarity and [EMIM][Ac] had high polarity. 150 mg lyophilized collagen was added to 30g those two ILs respectively. The concentration of soluble collagen in 70% [EMIM][ $\text{BF}_4$ ] was just 0.02 mg/ml and that of collagen in [EMIM][Ac] increased from 1.94 for 10% [EMIM][Ac] to 3.57 mg/ml for 70% [EMIM][Ac]. Td of the insoluble collagen fibers treated with 70% [EMIM][ $\text{BF}_4$ ] was 61.2°C similar to the native collagen fibers (61.9°C) and that of fibers treated with 70% [EMIM][Ac] decreased to 50.6°C. The collagen fibers treated with [EMIM][ $\text{BF}_4$ ] were looser than the native ones, while the fibers derived from [EMIM][Ac] were much more sparsely distribution, as shown in Fig. 5. In the high polarity IL, the collagen fibers would become looser and easier mobile, resulting in the reducing of thermal stability. The thermal stability and fibril-formation of collagen (grass carp skin) in three EMIM-based ILs with anions of bromide (Br), chloride (Cl) and acetate (Ac) were reported by Zhai et al. [151]. The collagen solution was mixed with equal volume of phosphate buffer (pH 7.4) containing one of the three ILs (0.05 M) and was kept the final collagen concentration at 1 mg/ml. The native collagen solution and the solutions treated with the ILs were incubated at 30°C for 2h to be further determined by DSC. The solutions showed a typical native collagen CD spectrum. The Td values of native collagen fibers and the fibers treated with [EMIM][Br], [EMIM][Cl] and [EMIM][Ac] were 38.86, 39.47, 40.38 and 50.9°C respectively. The diameters of collagen fibers showed a gradually upward trend in the order of  $\text{CH}_3\text{COO}^- > \text{Cl}^- > \text{Br}^- > \text{native collagen}$ , which were  $109.4 \pm 32.6$ ,  $96.5 \pm 20.8$ ,  $91.8 \pm 18.8$  and  $75.8 \pm 14.3$  nm respectively. The three ILs could promote the fiber-formation and then increased the thermal stability of collagen fibers.

Collagen is an amphoteric charged protein. Meanwhile, various types of ILs would exhibit a number of distinct properties such as polarity, hydrophobicity and hydrogen-bonding capability. The interaction between ILs and collagen might be highly specific and quite complex in nature. The structure and thermal stability of collagen would be vast affected by the different combination of the cations and anions in ILs.

#### 4.5 Alcohols

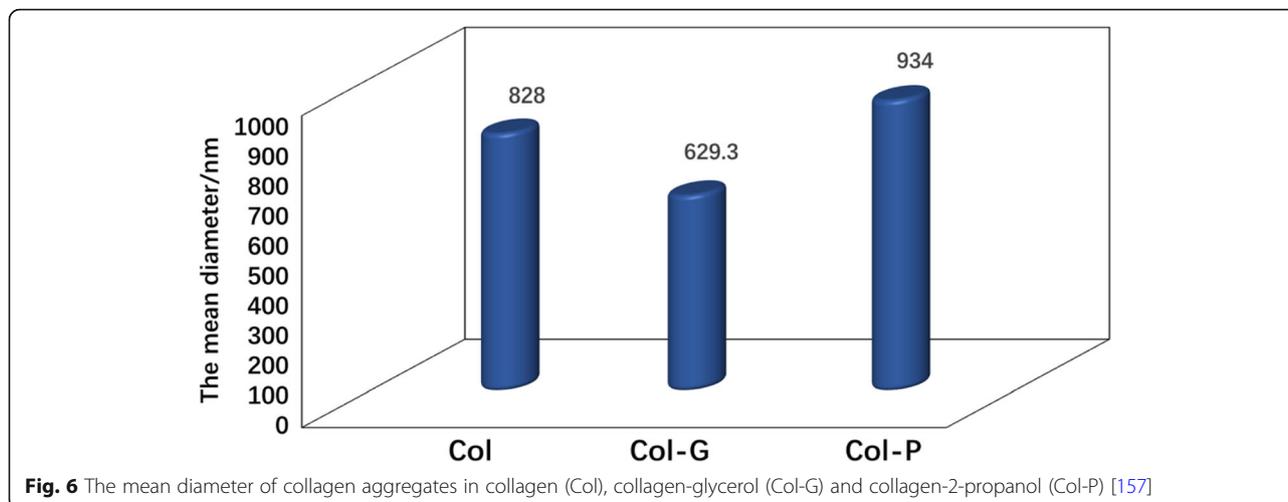
In aqueous environment, collagen could fold into the most stable conformation through hydrophobic interactions among nonpolar amino acid residues. Additionally, the repulsive forces between water and nonpolar side chains of collagen could favor the ordering of water structure to stabilize the collagen [152]. The thermal stability of collagen was greatly affected by adding alcohols which included glycerol, monohydric alcohols and dihydric alcohols.



The effect of glycerol (Gly) on collagen was studied by Penkova et al. [153, 154]. 0.5 mg/ml PSC-s of calf skin (CSC), sheep skin (SSC), RTT (RTC) and human placenta (HPC) were chosen to be mixed with glycerol of different concentrations (0–4M). Td of the samples was positive linear dependent with the glycerol content. The equations were given as  $Td = 40.45 + 1.15 \times [Gly]$  (CSC),  $Td = 40.68 + 0.84 \times [Gly]$  (SSC),  $Td = 40.46 + 0.71 \times [Gly]$  (RTC),  $Td = 40.74 + 0.95 \times [Gly]$  (HPC). The collagen was proposed to preferred to interact with Gly rather than water. Every individual polypeptide  $\alpha$ -chain of collagen was stabilized through hydrogen bonding which was formed between the hydroxyl groups of Gly and Hyp residues within two neighboring triplets [153]. The effect of Gly on collagen during denaturation was further studied. 0–3 M Urea and 0–0.15 M NaCl were chosen as the reagents of destabilizing collagen. The effect of those two reagents destabilizing collagen were reduced owing to the presence of the Gly. The dependence of Td on the urea and sodium chloride concentration in the presence of 0–3M Gly showed a set of parallel lines which were shifted upward by about 1°C per 1M glycerol [154]. The solvation shell surrounding collagen molecules were stabilized, because Gly with greater ability of forming hydrogen bonds was preferential binding with water [155]. The collagen was stabilized by Gly.

The thermal stability and aggregation behavior of collagen in Gly solution and 2-propanol solution were studied by Li et al. [156, 157]. Lyophilized collagen of calf skin was dissolved into 0.1 M acetic acid containing 0.5–3 M Gly or 2-proanol. The final collagen concentration was kept at 1mg/ml. The transition temperature (Tm) of the collagen was positive linear dependent with the concentration of Gly but was negative linear dependent with the concentration of 2-proanol. The equations were given as  $Tm = 41.45 + 0.929 \times [Gly]$ ,  $Tm = 41.44 - 1.638 \times [2-proanol]$ . Additionally, the mean diameter of collagen

aggregates in 2-propanol showed higher and that of in Gly exhibited lower than that of the pure collagen aggregates, as shown in Fig. 6. The intermolecular aggregation was induced by the increase of hydrophobic residues in 2-propanol solution. The aggregation behavior of collagen was hampered in Gly due to the interaction between Gly and collagen. Aliphatic alcohols could promote the solubility of hydrophobic residues of collagen into the solvent medium to weaken hydrophobic bonds, thus destabilizing the triple helix [158, 159]. The effect of monohydric alcohols with different specific dielectric constant ( $\epsilon_r$ ) on the collagen was reported by Nezu et al. [160]. Water has the  $\epsilon_r$  value of above 80 and many organic compounds have smaller  $\epsilon_r$  values. The lower  $\epsilon_r$  value of alcohols, the lower affinity between water and alcohols. Methanol (n-C<sub>1</sub>OH), ethanol (n-C<sub>2</sub>OH), 1-propanol (n-C<sub>3</sub>OH) and 1-hexanol (n-C<sub>6</sub>OH) were chosen, whose  $\epsilon_r$  values were 33.0, 25.3, 20.8 and 13.0 respectively. Calfskin collagen was added with the alcohols of different concentrations (wt%) to obtain a constant collagen concentration at pH 3.0. Precipitation of collagen from solution would occur in a higher concentration of solvents [161], thus the thermal behavior of collagen was observed mainly in low concentrations of monohydric alcohols (<40%). The Td values of collagen solutions decreased with the increase of monohydric alcohols concentration. The tendency was more evident in the alcohols of a lower specific dielectric constant ( $\epsilon_r$ ). Td of the collagen in 20% n-C<sub>1</sub>OH, n-C<sub>2</sub>OH and n-C<sub>3</sub>OH decreased by about 1, 4 and 7°C respectively. The hydrophobic interactions among collagen molecules were weakened as a more hydrophobic alcohol was added, which led to a lower Td. Gopinath et al. [162] investigated the effect of ethanol on the triple helix of collagen. 4 mg/ml collagen (RTT) stock solution was prepared by dissolving lyophilized collagen into 50 mM acetic acid. The final collagen solution (0.4 mg/ml) was



**Fig. 6** The mean diameter of collagen aggregates in collagen (Col), collagen-glycerol (Col-G) and collagen-2-propanol (Col-P) [157]

prepared through adding appropriate volumes of ethanol of different concentrations and 50 mM acetic acid into collagen stock solution (4 mg/ml). The collagen solution gradually turned into a gel in the ethanol with concentration above 50% due to the dehydration effect of ethanol. Therefore, collagen solutions in 0–40% ethanol were further studied. Melting temperature ( $T_m$ ) of the solutions was determined by CD. The  $T_m$  values of collagen gradually decreased from 40 (0% ethanol) to 34°C (40% ethanol). The characteristic CD spectrum of native collagen could be observed from the solutions. The molar ellipticity at 220nm in the presence of ethanol (10%–40%) was higher than that in the absence of ethanol. The collagen molecules became closer owing to the dehydration effect of ethanol, which could further disrupt the water network surrounding collagen monomers and water-mediated hydrogen bonds among collagen molecules. The change of molar ellipticity of collagen at 220nm in various alcohols was studied by Usha et al. [163]. RTT collagen was mixed with 0.05–0.2 M methanol and ethanol respectively. 0.2 M Gly, n-propanol and propane 1,2-diol were added into collagen solution respectively as well. The collagen of constant concentration ( $2 \times 10^{-6}$  M) was measured by CD. The molar ellipticity at 220 nm of collagen increased with the increase in concentration of methanol and ethanol and that of the collagen in 0.2M Gly, n-propanol and 1,2-propanediol was lower than that of the pure collagen. The reason is still not clear. The collagen molecules would become closer and the water-mediated hydrogen bonds among collagen molecules might be destroyed in methanol and ethanol due to dehydrating effect. The hydrophobic residues of collagen would be more easily exposed in solvent after adding an alcohol of a lower polarity, which causes the secondary structure of collagen to become loose. Both mechanisms could make the triple helix require less energy to unfold during heating.

Collagen shows a lower thermal stability in monohydric alcohols.

The effect of a number of substituted diols on the thermal stability of collagen was investigated by Hart et al. [164]. A set of diols with different hydrocarbon chains and hydroxyl position included ethylene glycol, propan-1,3-diol, propan-1,2-diol, butane-1,4-diol, butane-1,3-diol, pentane-1,5-diol and hexane-1,6-diol. Calfskin collagen solution was added with these diols respectively and kept a constant collagen concentration for further determining melting temperature ( $T_m$ ) by optical rotation.  $T_m$  of the collagen in ethylene glycol and propan-1,3-diol increased and that of the collagen in the other diols decreased as the concentration of diols increased. The decreased tendency of  $T_m$  became more obviously as the hydrocarbon chains of diols lengthened. The collagen could be stabilized in the hydroxyl-terminated diols with short chain, probably because the diols similar to Gly have the ability of forming hydrogen bonds with Hyp residues of collagen. The stabilization of collagen could not be obtained in propan-1,2-diol, speculating the reason is that the two hydroxyls are too close to form “link” among collagen molecules. The thermal stability of collagen kept decreasing as the hydrocarbon chains lengthened, because the nonpolar groups which dominated in a longer chain diol promoted the exposure of hydrophobic residues within collagen chains into solvent.

### 5 The effect of blending with polymers on thermal stability

The disadvantages of collagen, such as low thermal stability, weak mechanical property and poor water & enzymatic degradation resistance, would limit its applications. The blending of collagen and other polymers is the easiest way to obtain collagen-based biomaterials of improved properties. Natural polymers and

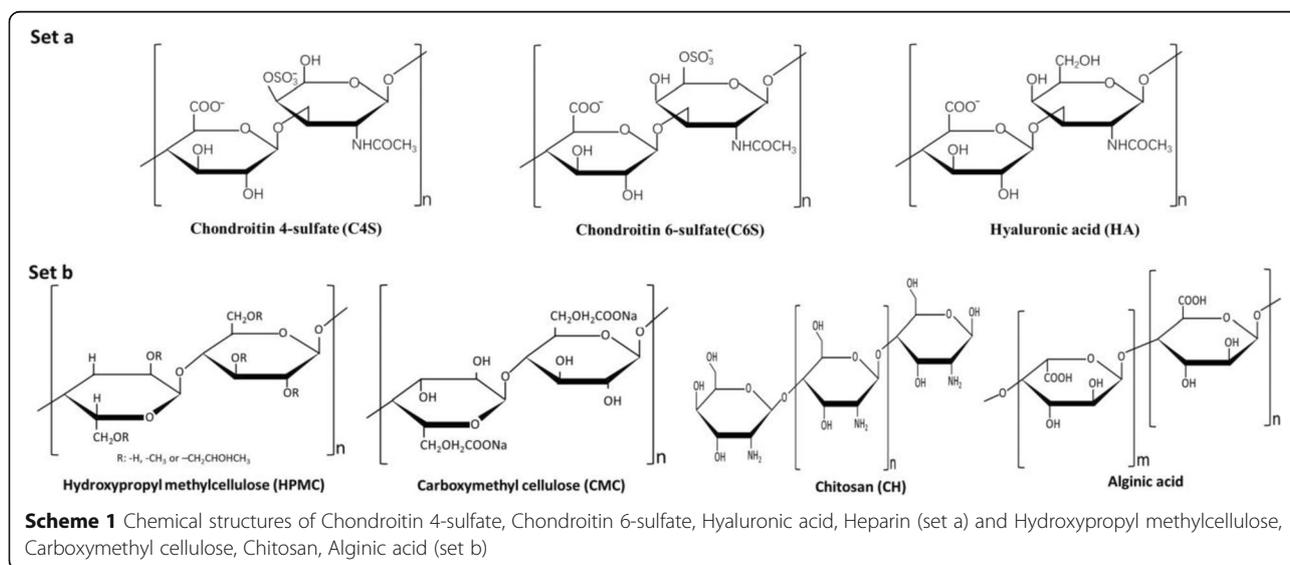
biocompatible synthetic polymers with desirable biological features and mechanical properties are usually used to blend with collagen. The blends are usually prepared in solution and then manufactured into hydrogels, scaffolds, sponges, nanofibers or films. The thermal stability and miscibility of the blends in solution are important for further processing. The miscibility is judged by the comparison of the specific viscosity ( $[\eta]_{exp m}$ ) and the ideal intrinsic viscosity  $[\eta]_{i m}$  of the blends. The blends are compatible as  $[\eta]_{exp m} > [\eta]_{i m}$  and are incompatible as  $[\eta]_{exp m} < [\eta]_{i m}$  [165].

### 5.1 Natural polymers

As natural polymers with excellent biocompatible and biodegradable properties, Chondroitin sulfate (CS), Hyaluronic acid (HA), Hydroxypropyl methylcellulose (HPMC), Carboxymethyl cellulose (CMC), Chitosan (CH) and Alginic acid have been commonly used to blend with collagen. The chemical structure of those nature polymers is presented in Scheme 1. CS and HA as linear polyanions belong to the class of macromolecules known as glycosaminoglycans (GSG). Cellulose, CH and Alginic acid belong to polysaccharide with a highly ordered chemical structure. All those polymers contain abundant hydroxyl groups. Except for HPMC and CH, the others contain negatively charged group such as sulfo and/or carboxyl groups, which easily form precipitate with positively charged collagen in acid solution due to the strong electrostatic interaction. The charge shielding is necessary to obtain a miscible binary blend. The addition of NaCl is an effective approach.

The properties of the blends of collagen (Col) with CS or HA and the interaction between collagen and CS or HA were studied by Li and her groups [166–169]. Lyophilized collagen and chondroitin 4-sulfate (C4S) were

dissolved in the phosphate buffered saline (PBS including 10 mmol/L phosphate and 100 mmol/L NaCl) at pH 7.4 respectively to prepare the stock solutions. The Col/C4S blends were obtained after mixing those two stock solutions at varying Col/C4S weight ratios of 100/0–20/80. The final collagen concentration was kept at 1 mg/ml by adding suitable PBS. The solutions were incubated at 37°C for 60 min to form cofibrils. The thermal stability of cofibrils increased with the increase of the C4S content. The maximum transition temperature ( $T_m$ ) of the cofibrils evidently increased from 42.7 to 53.9 at the Col/C4S ratios of 100/0–91/9 and then reached to 61.9°C at ratio of 70/30. The increased tendency became slow at a ratio  $> 50/50$  and  $T_m$  was 65.8°C at ratio of 20/80. The microfibrillar became large and order from small and disorder as C4S increased. Additionally, the D-periodicity of the cofibrils could be observed as ratios were 20/80 and 80/20. The self-assembly was carried out according to the original model and was accelerated after the addition of CS4, which promoted the thermal stability of cofibrils [166]. The properties of the blends of collagen and HA was also studied. Tris-HCl at pH 7.2 was used as the solvent. The blends were prepared at various Col/HA weight ratios of 8/2, 5/5 and 2/8 and then lyophilized.  $T_d$  of the sponges at ratios of 8/2, 5/5 and 2/8 were 56, 61.3 and 69.5°C respectively. The morphology of the sponge at ratio of 5/5 contained the least sheet structure and exhibited the most homogeneous comparing with that of the sponges as ratios were 8/2 and 2/8 [167]. The hydrogen bonds between collagen and CS or HA was observed in the further studies using FTIR (Fourier transform-infrared) and 2D correlation FTIR analysis. 0.25 mol/L NaCl at pH 7.4 was used as solvent. The Col/CS blends were prepared at Col/CS weight ratios of 100/0–9/91. The blends were



judged to be miscible, because  $[\eta]_{exp m}$  was always higher than  $[\eta]_{i m}$ . The evidence of interaction between collagen and CS came from the shift of the amide I band and the decrease of the intensity of amide II band of collagen as Col/CS ratios changed from 100/0 to 20/80. The hydrogen bond was formed between hydroxyl groups of CS and C=O group of collagen as the CS content was below 50 wt% because of the charge shielding effect of NaCl. When CS content was more than 50 wt%, the electrostatic interactions was formed between carboxyl or sulfate group of CS and amino group of lysine or guanidine group of arginine residues as well as hydrogen bonds between C=O group of CS and amino groups of collagen [168]. The similar measurement was used to analyze the interaction between collagen and HA. The blends of collagen and HA in 0.2 mol/L NaCl solution were prepared at the Col/HA weight ratios of 100/0–0/100 and then air-dried. The red-shift of amide I band and blue-shift of amide II band of collagen were observed from FTIR spectra, which demonstrated the existence of interaction between collagen and HA. The hydrogen bond between hydroxyl group of HA and C=O group of collagen would be formed as HA content increased from 0 to 50% and that between C=O group of HA and N-H group of collagen was generated as HA content was 50%–90% [169]. The physicochemical and biological properties of the blends might be related to the different Col/CS or Col/HA ratios at which the interactions between collagen and those two polymers were different.

Cellulose with highly hydrophilic property cannot be soluble in a common solvent owing to strong inter- and intra-molecular hydrogen bonds. As cellulose derivatives, HPMC and CMC of highly water-solubility are usually used to blend with collagen. HPMC is a typical nonionic polysaccharide and CMC is anionic. The properties of the collagen/HPMC blends were investigated by Li and her group [170–172]. The blend solutions were prepared by mixing 15mg/L collagen with 15mg/L HPMC solution (0.1M acetic acid as the solvent) at the Col/HPMC ration of 1/1 (w/w) and then air-dried at room temperature. The endothermic peak of the Col/HPMC film was 6°C higher than that of the collagen film. The tensile strength of the film increased by 7.1 Mpa comparing with the collagen film [170]. The thermal stability of Col/HPMC gels at different Col/HPMC weight ratios also be studied. PBS (pH 7.2) containing 100 mM NaCl was used as the solvent. The blends of collagen and HPMC were prepared at the ratios of 0/1–3/1 and the collagen concentration was kept at 1mg/ml. The blend solutions were then incubated at 37°C for 60 min. The thermal stability of hydrogels was determined by turbidity measurement at 46°C. The lower reduction of turbidity, the higher thermal stability. The values of reduction

in turbidity of the hydrogels mixed with HPMC were lower than that of the native collagen (49.8%), which indicated the thermal stability of Col/HPMC was increased. The thermal stability of hydrogels exhibited the tendency of increase before decrease and reached to the highest at ratio of 0.25/1 where the value of reduction in turbidity was 24.5% [171]. The interaction between collagen and HPMC varied with the different Col/HPMC ratios. The Col/HPMC blends were prepared by mixing the two solutions (in 0.1 M acetic acid solution) at the ratios of 10/0–0/10 and kept the collagen concentration at 5 mg/ml. The blends showed incompatible as the HPMC content was above 50% under which  $[\eta]_{exp m}$  was lower than  $[\eta]_{i m}$ . The hydrogen bonds between HPMC and collagen were observed in 2D correlation FTIR spectrum and became weaker at a ratio above 7/3. The hydrogen bonds tended to be formed among HPMC molecules, which resulted in weakening of interaction between collagen and HPMC [172]. Collagen whose isoelectric point is near physiological pH could not be dissolved in neutral solution. The acylation of collagen could solve the problem through converting the amino groups of lysine residues to carboxy groups [173]. Zhang et al. [174] used the deionized water as the solvent to blend succinylated collagen (SC) and CMC at different ratios of 10/0–0/10 (v/v). The blends were incompatible ( $[\eta]_{exp m} < [\eta]_{i m}$ ) as the ratios were 3/7 and 1/9. The denaturation temperature of blends increased by 0.9°C at ratio of 5/5 and then decreased by 0.2°C and 0.5°C as ratios were 3/7 and 1/9 respectively comparing with SC. The hydrogen bonds and electrostatic interactions between SC and CMC could improve the stability of the blends as the content of CMC was below 50%. The interaction between SC and CMC would gradually be weakened with the increase of CMC content due to preferential formation of hydrogen bonds among CMC molecules, which decreased the thermal stability of the blends.

CH containing free amino groups is the only alkaline natural polysaccharide. Fu et al. [175] studied the properties of collagen/CH membranes. The blends solution could be obtained by mixing 3 mg/ml collagen solution and 2% CH at virous volume ratios of 4/1–1/4 and then air-dried. Td of the membranes at Col/CH ratio of 4/1 was 10°C higher and that of the membranes at other ratios was lower than that of the native collagen membrane. When CH content was above 40%, the collagen fibers became remarkably loose and CH evenly distributed around collagen fibers observed in AFM images. Sionkowska et al. [176] using X-ray diffraction detected the helix structure in the collagen/CH films gradually was lost as CH content increased. 0.5M acetic acid was used as the solvent in the study. The blends were prepared to contain different collagen contents of 0–100% and then dried in vacuum at room temperature. The

solutions were miscibility, because  $[\eta]_{exp}$  were always higher than  $[\eta]_{im}$ . The characteristic peaks of native collagen in the solutions, which were observed by X-ray diffraction, gradually disappeared as the CH content increased. The hydrogen bonding was formed between -OH groups of CH and -NH<sub>2</sub> groups of collagen as well as between the end groups (-COOH and -NH<sub>2</sub>) of collagen and -OH and -NH<sub>2</sub> groups of CH. The appearance of new hydrogen bonding networks was speculated to alter the collagen helical character, thus affecting the thermal stability.

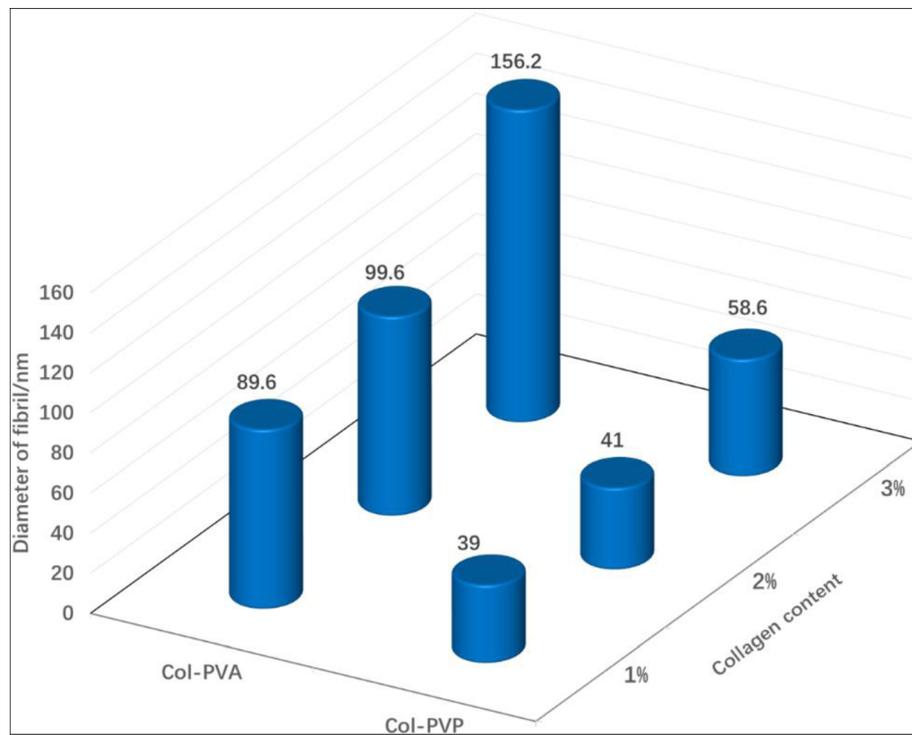
Alginic acid is a linear anionic copolymer arranged as homopolymeric or heteropolymeric block. In Mitra's study [177], alginic acid was regarded as a potential "cross-linker" for collagen. 0.5% collagen of bovine skin was added with alginic acid solution of different concentrations (PBS, pH 6.5) at the Col/Alginic acid ratio of 3/1. The blend solutions were incubated for overnight at 4°C and then air-dried at 37°C for 12h. The solution of Glutaraldehyde (1.5%) crosslinking collagen was carried out as a comparison. As alginic acid concentration was 1.5%, the melting temperature of the films gradually increased to 150°C, which was similar to the comparison (151°C) and much higher than that of the native collagen film (96.98°C). The crosslinking degree kept constant after adding alginic acid at a concentration above 1.5%. The biologically active lactone ester was proposed to be formed in alginic acid due to loss of water during film formation and then reacted with free-NH<sub>2</sub> groups of lysine residues in collagen chains, which formed chemical crosslinks among collagen molecules to improve the thermal stability. The thermal stability decreased as alginic acid concentration was further increased. Melting temperature shifted towards left might be caused by unreacted alginic acid whose melting temperature was about 90°C.

## 5.2 Biocompatible synthetic polymers

Water soluble synthetic polymers with desirable biocompatibility, physiological inertia and plasticity, such as Poly-vinyl-alcohol (PVA), poly-vinyl-pyrrolidone (PVP), poly-ethylene-glycol (PEG) and poly-D, L-lactide-co-glycolide (PLGA), have broad range of applications. The thermal stability and properties of collagen blending with those polymers have been investigated. Collagen are commonly blended with PVA and PVP which have been widely applied in various fields. The properties of Col/PVA blends were studied by Lai et al. [178]. 2.1wt% blends were obtained after mixing the collagen and PVA solution at different Col/PVA weight ratios and then lyophilized. Dynamic denaturation temperature of the blends increased from 33.0°C for 100/0 to 35.6°C for 20/80. The interaction between collagen and PVA showed the strongest as the contents of PVA were 20–30%,

which was demonstrated by analysis of FTIR. The formation of hydrogen bonds between collagen and PVA would be weakened at a higher content of collagen or PVA. The miscibility of collagen and PVA was reported by Sarti and Scandola [179]. The Col/PVA complexes with different PVA contents were air-dried at room temperature to obtain the films. Td of the native collagen film was 117°C and the glass transition temperature (Tg) of PVA was 50°C. The Td values of the films showed an upward tendency with the increase of PVA content and increased by 14°C at PVA content of 70%. Two distinct peaks of the Col/PVA films could be evidently observed in DSC curves, especially when the contents of PVA were 70% and 50%, which accorded with Tg and Td of individual compounds. The blends of collagen and PVA was proposed to be partly miscible. The properties of Collagen/PVP were studied by Sionkowska [180]. 0.4 M acetic acid was used to dissolve collagen and PVP. The blends at Col/PVP weight ratios of 20/80–80/20 were demonstrated to be miscible ( $[\eta]_{exp} > [\eta]_{im}$ ). The first peak (T<sub>1</sub>) of DSC for collagen/PVP film (87°C) at ratio of 50/50 was higher than that of the single components (collagen was 80°C and PVP was 82°C), where T<sub>1</sub> attributed to unfolding of triple helix for collagen and the single glass transition temperature for PVP respectively. The strong hydrogen bonds were formed between the carbonyl groups of the pyrrolidone rings in PVP and the hydroxyl and/or amino groups of collagen, which caused the collagen and PVP to be miscible. Additionally, the diameter of collagen fibrils in PVA film was larger than that in PVP at the same condition, as shown in Fig. 7. The weak interaction between collagen and PVA resulted in partially miscible, which did not disturb in collagen fibril formation. The strong interaction between collagen and PVP hampered the collagen molecules to form fibrils [181]. The thermal stability of Col/PEG sponge was studied by Xue et al. [182]. 1% collagen solution (5 mol/L acetic acid) was added with PEG-20000 of different concentrations and then lyophilized. Td of the native collagen was 68.5°C and Tg of PEG was 67.5°C. The Td values of Col/PEG films were first decreased to 59.7°C (10% PEG) and then increased to 65.1°C (50% PEG). The decrease of thermal stability was also observed in the blends of collagen and PLGA [183]. Collagen and PLGA were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP). The blends were obtained with different Col/PLGA ratios of 50/50, 35/65 and 20/80 and then used for spinning. Td of the native collagen was about 80°C and Tg of PLGA was 42°C. The Td values of complex decreased with the increase of the PLGA content. The blends were suggested to be immiscible due to the appearance of two distinct peaks of DSC curves.

Other biocompatibility polymers have also been used to blend with collagen for biomedical applications. The complex materials are usually manufactured into the



**Fig. 7** Diameter of collagen fibrils in PVA (Col-PVA) and PVP (Col-PVP) films containing 1%, 3% and 5% of collagen respectively [181]

form of hydrogel, film and scaffold which have much higher thermal stability due to loss of free water. Mechanical and biological properties of the blends have always been taken a major concern. The ratio of collagen to polymer would affect the physicochemical properties of the blends and should adapt to practical demand.

## 6 The effect of crosslinking on thermal stability

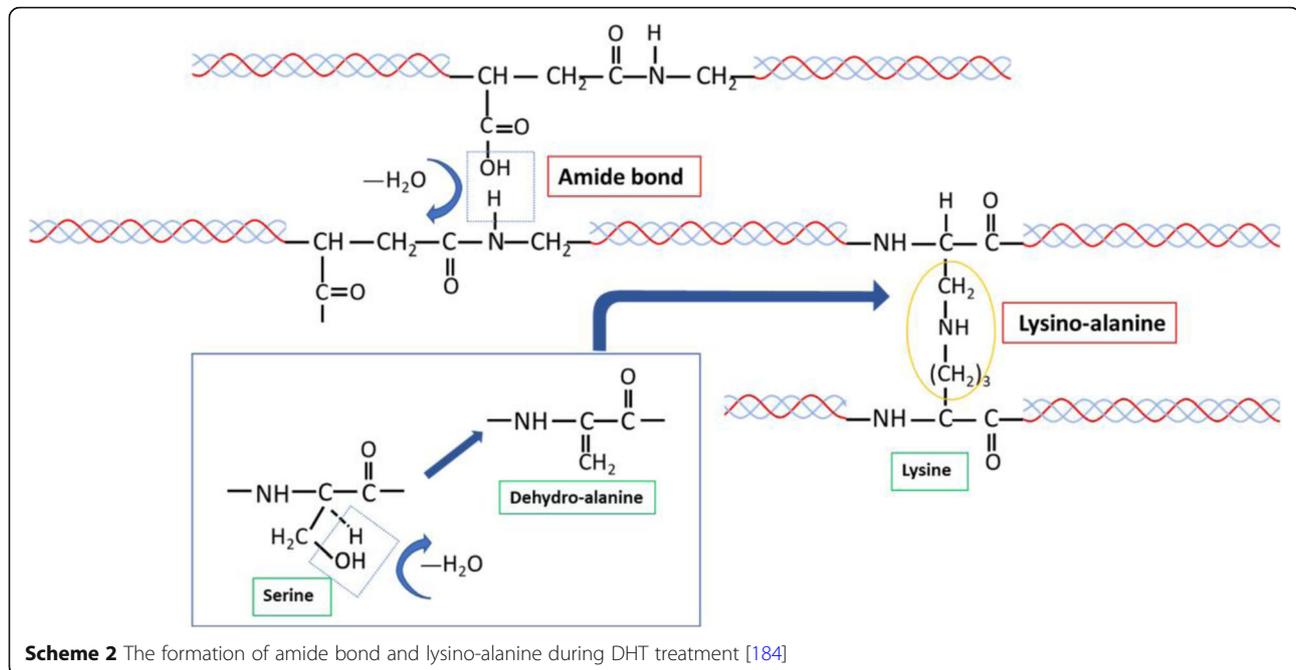
Crosslinking as the best effective method is generally used to promote collagen's thermal stability, mechanical property and resistance to enzymatic degradation. A crosslinking could be defined as the induction by physical or chemical method to connect the functional groups within one collagen chain to that in another chain. Herein, on the view of thermal stability, the physical and chemical crosslinking of collagen are summarized.

### 6.1 Physical crosslinking

Physical crosslinking is accepted as a safe, cheap and simple modification owing to no additional chemical reagents. Dehydrathermal (DHT) treatment, ultraviolet (UV) irradiation and ionizing radiation (gamma-ray and electron beam) are generally used.

DHT treatment is a commonly recommended approach of physical crosslinking, which is performed on heating dry collagen matrix under vacuum to around 100°C for 24–72 h. During DHT process, the formation of the amide bonds between amine and carboxyl groups

of collagen as well as the formation of the lysino-alanine bonds between the dehydro-alanine (produced by  $\beta$ -elimination of serine residues) and lysine residues in collagen chains cause intra- and inter-molecular crosslinks, as shown in Scheme 2 [184]. An appropriate DHT treatment is positive to thermal stability of collagen. The spinning collagen fibers were dried under tension at room temperature for 16 h and then heated in a vacuum oven at 110°C for 72 h. The shrinkage temperature of bovine skin collagen fibers after DHT treatment increased by 12°C comparing with the uncrosslinked fibers, as reported by Weadock et al. [185]. The thermal stability of the porcine acellular dermal matrix scaffolds was also improved after DHT treatment [186]. The scaffold was obtained after lyophilizing the porcine skin which was removed the non-collagenous substances. The residual water continued to be removed at 40°C, 0.05 bar for 6 h. Crosslinking was carried out at 110°C for 12 h. The Td values of the crosslinked scaffold was 6°C higher than that of uncross-linked scaffold. The thermal stability of fish skin collagen film was not insignificantly improved after DHT treatment. 0.5% silver carp collagen solution was air-dried under a laminar air-flow at room temperature. Td of the crosslinked film after heating at 105°C for 24 h under vacuum (0.05 Bar) was merely 3°C higher than that of non-crosslinked film [187]. Gorham and Light [184] found thermal degradation would take place as the heating temperature



increased. 0.3% collagen solution (1–2 years old bovine skin) was lyophilized and then heated at different temperatures for 24 h under a vacuum of 11 mbar. Td of the collagen without heat treatment was 66.3°C. The Td values of crosslinked collagen increased to 66.2°C as the heating temperature was 60°C and then decreased as the heating temperature further increased. The characteristic  $\alpha 1$ ,  $\alpha 2$  and  $\beta$  chains of native collagen were observed in SDS-PAGE to be degraded into low molecular peptides as the heating temperature was 120°C. However, the lysino-alanine bonds (crosslink bonds) increased as the heating temperature rose. The crosslinking and denaturation occur simultaneously during DHT treatment. The parameters of DHT treatment should be adjusted according to the different sources of collagen and the state of samples. Dehydration and drying of collagen samples are necessary prior to DHT treatment to prevent degradation under the high temperature. The thermal stability of collagen would significantly increase with the decrease of intra-fibrillar water content in collagen. Schroeffer and Meyer [188] determined the denaturation temperature of bovine hide at varying humidity by DSC. The humidity was adjusted by placing a small piece of hide under saturated water vapor or in a desiccator or by directly adding water. The Td values of hide decreased from about 180°C at water content of 3.7% to about 60°C at water content of 44%. Td would not decrease as a water content was above 44% at which the collagen fibers were fully hydrated. The thermal stability of collagen could be improved by dehydration drying. Shen et al. [189] prepared a high thermal stability fish

(southern catfish) collagen gel through the dehydration of ethanol. Collagen solution (5 mg/ml) was incubated at 30°C for 5 h as a control. The improved gels were obtained after being immersed in different concentrations of ethanol solutions (20–100%) for 24 h at collagen/ethanol ratio of 1/10 (w/v). Td of the gels slowly increased from 43.1°C (control) to 49.2°C (60% ethanol) and then evidently increased to 60°C (80% ethanol). Td of the gel could reach to 75.8°C in 100% ethanol solution.

UV irradiation as a non-ionizing irradiation could induce the amino acids residues (such as Trp, Phe and Tyr) of collagen to generate free radical to cause intermolecular crosslinks. Sinokowska and his group [190, 191] investigated the effect of sample state and UV irradiation time on the thermal stability of collagen. Three forms of collagen were prepared using RTT collagen. Tendon pieces were obtained from rat tail and then dried at 35°C. Collagen solution was prepared by dissolving tendon fibers into 0.04 M acetic acid. Collagen film (0.015 mm) were obtained by air-drying the collagen solution at 35°C. The samples were irradiated at room temperature for different duration time. Td of the solution was determined by viscosity measurements and Td of pieces and film were determined by DSC. Td of the native solution, tendon piece and film were 40.2, 101.9 and 110.2°C respectively. The Td values of samples decreased as the irradiation time lengthened. After irradiating 2 h, Td of the solution could not be determined due to completely denaturation. The Td values of piece and film decreased by 16.3°C and 4.3°C respectively [190]. The effect of irradiation on the thermal stability of RTT

fibers in aqueous was also studied, which was carried out through immersing the RTT fibers in water and then irradiating at room temperature under air atmosphere for varying time. Td increased from 64.1°C to 67°C after irradiating for 0–3h and then decreased from 57°C to 39.3°C after irradiating time was lengthened from 20 to 66 h [191]. The thermal stability of collagen crosslinked using UV irradiation was depended on the hydration degree of sample and the dosage of incident radiation (= intensity × time).

Exposure of collagen to ionizing irradiation such as gamma ray and electron beams is the other way to crosslink collagen through free radical mediation. Usually, hydroxyl radicals generated by the amino acid residues such as Phe, His and Met residues in collagen after absorbing irradiation form intermolecular crosslinks [192]. Tyrosine residues is also proposed as a crosslinking point. Crosslinking is carried out by bonding two tyrosyl radicals to form dityrosine bonds [193]. Inoue et al. [194] prepared collagen gels by directly irradiating collagen solution in acidic condition using gamma-rays. Type I collagen of porcine was dissolved into solution at pH 3.0 to obtain acid collagen solution (0.3%). The acidic gels were obtained after irradiating with gamma-rays of different doses of 0–31 kGy (dose rate 15–16kGy/h) at room temperature in air. Neutral collagen solution was obtained by adjusting the pH value with PBS (pH 7.4) and then incubated at 37°C for 30 min. The neutral gels were irradiated at same condition as acidic gels. Acidic gels were transparent and highly shrank while neutral gels kept original state as the irradiation dose increased. Crosslinking rather than degradation was proposed to occur in acidic gels under the low dose of gamma-rays irradiation (1.4 and 2.1 kGy), because the  $\alpha 1$  and  $\alpha 2$  chains of the acidic gels molecules shifted to a higher molecular weight and there were not any fragmented collagen with lower molecular weight observed in SDS-PAGE. Acidic gels were gradually degraded as the irradiation dose further increased. Collagen gels prepared in aqueous solution by gamma-irradiation were studied by Zhang et al. [195]. 1.54% type I collagen solution (RTT, Beijing Kelaode, pH6.2) was directly irradiated with different dose (dose rate was 20 kGy/min) at room temperature to form collagen gels. The collagen gels were transparent and kept almost same size as irradiation doses were 10–20 kGy. The Td values of collagen gels irradiated with gamma-rays were higher than that of un-irradiated gel (66.4°C), which were 83.5 (5 kGy), 73.6 (10 kGy), 71.7 (15 kGy), 81.1 (20 kGy) and 89.8°C (25 kGy) respectively. Jiang et al. [196] used electron beams to modified calfskin collagen membrane which was obtained by drying self-assemble collagen fibers. The membranes were irradiated by electron beams at low temperature in nitrogen atmosphere with

different irradiation dose or without. The hydrothermal and xerothermic shrinkage temperature of the samples were determined by micro-shrinkage tester. The shrinkage temperature of the membranes increased with the increase of the irradiation dose and were about 15°C (100 kGy) higher than that of the un-irradiated membrane.

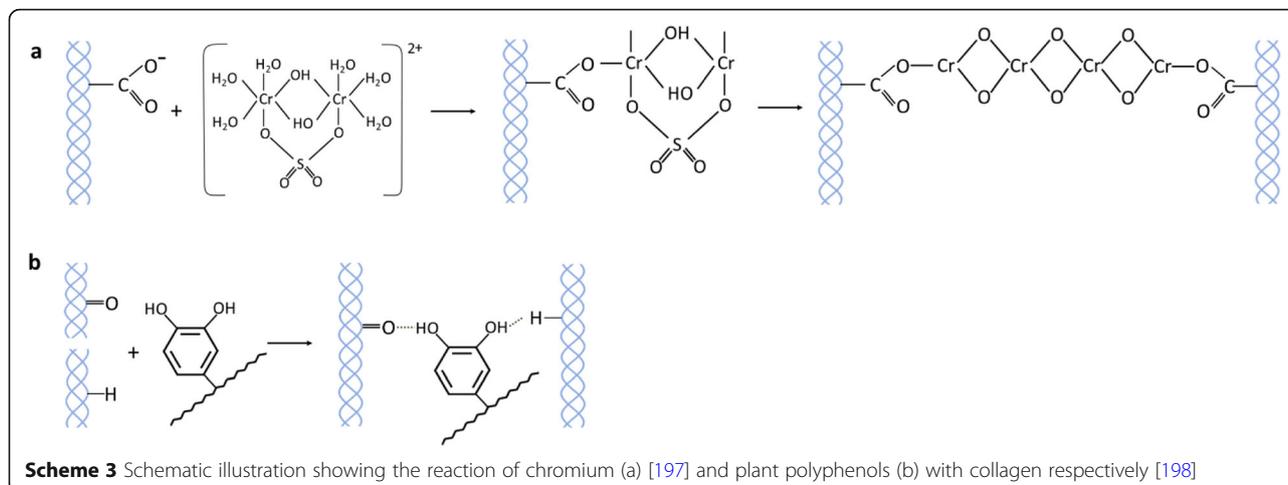
Obviously, physical crosslinking is affected by the state of sample, temperature, radiation dose, pH value and atmosphere condition. Crosslinking induced by physical method is always together with degradation which should be prevented by fine tuning of physical processes.

## 6.2 Chemical crosslinking

Chrome, plant tannin and aldehyde have been widely used in tanning to obtain leathers of various desirable properties such as high hydrothermal stability, favourable biological inertness and excellent mechanical property. Nowadays, more chemical reagents have been used to crosslink native collagen or collagenous materials for versatile applications. Chemical crosslinking is carried out via introducing functional groups into collagen molecules. Metal complex compounds, which are mainly applied to tanning for leather industry, stabilize collagen by forming coordinative bonds with the carboxylate groups of collagen chains. Plant polyphenols, as one of the commonly tanning agents as well as a crosslinker, crosslink collagen mainly through forming multipoint hydrogen bonds. Most of other chemical reagents crosslink collagen through different crosslinked mechanisms, which are mainly involved in the  $\epsilon$ -amino groups of collagen.

### 6.2.1 Metal complex compounds

Metal complex compounds crosslink collagen through coordinating with the carboxylate groups of collagen. Chromium (III) as the most commonly used tanning agent could remarkably improve the hydrothermal stability and chemical properties of leather. Ts of chrome-tanned leather could easily achieve to 110°C or higher. At least two carboxylate groups of collagen replace ligands (mainly water) of the polynuclear chrome complex to form stable coordination bonds, as shown in Scheme 3a [197]. At the same condition, the Ts values of the leathers tanned with chrome, zirconium and aluminum were 102, 83 and 72°C respectively [199]. Chromium is not suitable to crosslink collagen for other applications, because chromium is cytotoxic and might turn into hexavalent chromium of highly toxic. Zirconium, which is only second to chromium to promote the hydrothermal stability of leather, has good biocompatibility and low toxicity [200]. Therefore, zirconium is a candidate crosslinker to improve the properties of collagenous materials used in biomedicine. Zirconium is easily hydrolyzed and only stable at a low pH (< 3). Masking agents are usually added to modified the properties of zirconium complex. Liu et al. [201] used zirconium



complex modified by sodium citrate to crosslink calfskin collagen sponge which was obtained after lyophilizing collagen solution. Taking the denaturation temperature (by DSC) as the index, the optimal crosslinked condition was carried out: the molar ratio of sodium citrate/ zirconium (IV) was 0.18, crosslinked time was 2 h, the concentration of sponge in complex solution was 0.01 mol/L, temperature was 37°C. Td of the crosslinked sponge was 36.5°C higher than that of the uncross-linked sponge.

### 6.2.2 Plant polyphenols

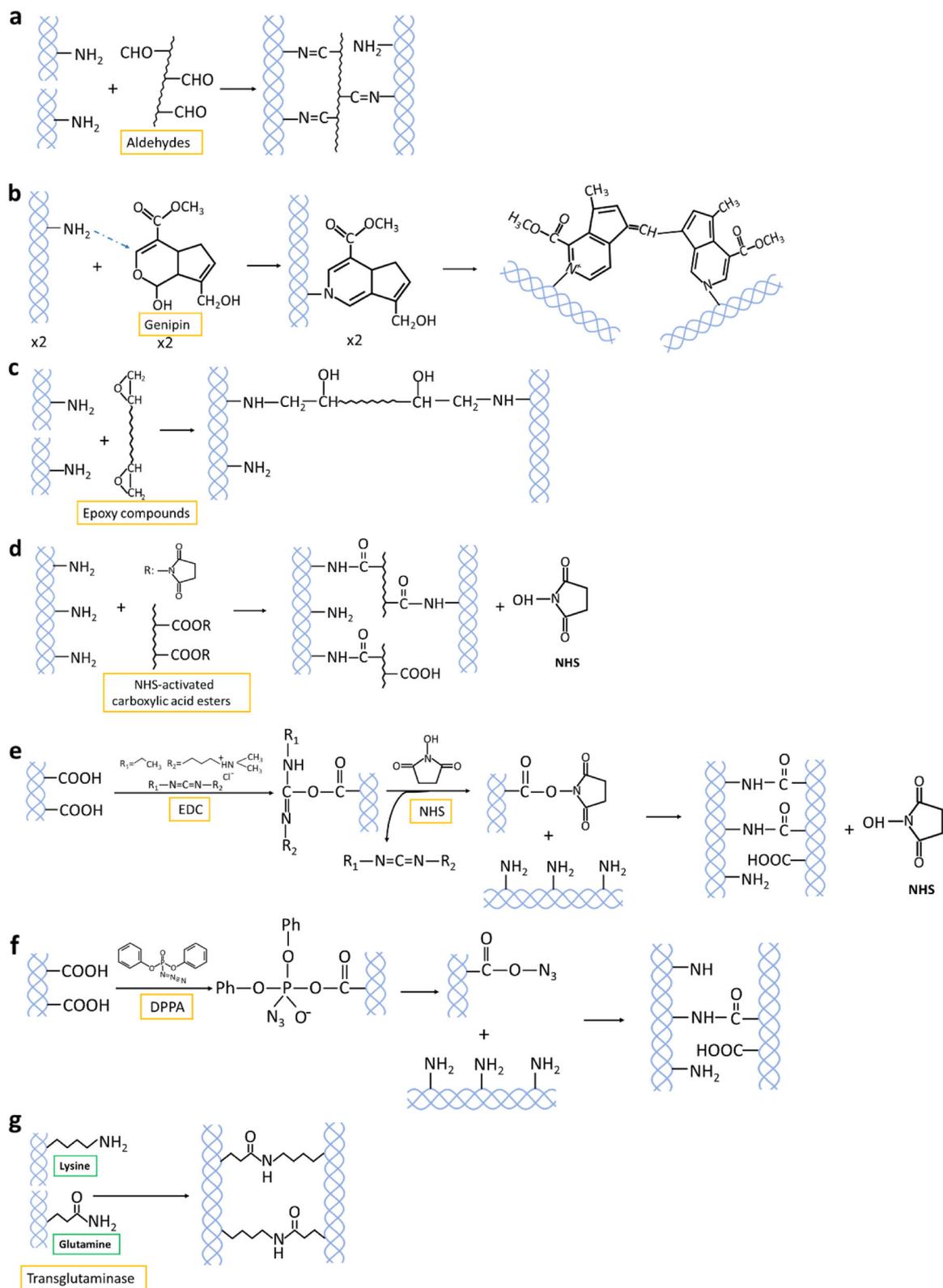
Plant polyphenols, also known as vegetable tannins, were firstly applied in leather industry. As a biomaterial, plant polyphenols have been used to modify collagen for more extensive applications. Multipoint hydrogen bonds could be formed between phenolic hydroxyl groups of polyphenols and hydroxyl, carboxyl, amino or amide groups of collagen chains, as shown in Scheme 3b [198]. He et al. [202] used procyanidin to crosslink collagen in solution. Collagen solution and procyanidin solution were mixed at different procyanidin/collagen ratios of 0–8% (w/w) at pH 3.0. The collagen concentration was kept at 2.5 mg/ml. Then the crosslinked mixtures were dried at room temperature for 1 week. Td of the native collagen films was 65.5°C and melting temperature of procyanidin was 70.0°C. The Td values of crosslinked films first increased and then decreased. Td of the film at procyanidin content of 4% was the highest (83.3°C) and then reduced to 74.3°C as procyanidin content was 8%. Madhan et al. [203] crosslinked RTT fibers through soaking the RTT fibers obtained after the removal of non-collagenous substances into the catechin solutions of different concentrations at 27°C for 27 h. The hydrothermal stability of the samples was determined by microshrinkage tester. The Ts values of the crosslinked fibers increased with the increase of catechin concentration and increased by 9°C as 20 mM catechin was added.

Catechin with crosslinking effect was also used as natural dyestuff to dye collagen membrane [204]. Catechin contains a large number of benzene rings and phenolic hydroxyl groups which are easily oxidized to form quinonoid chromophoric groups and also easily form conjugated fused rings to make polyphenols appear color. The best properties of membrane could be obtained after agitating in 2% catechin for 3h at 40°C (pH 4). Td of the membrane was 18°C higher than that of the native collagen membrane and the tensile strength of the membrane increased by 1.26 MPa after being crosslinked.

### 6.2.3 Other crosslinkers

The functional groups of most crosslinkers such as aldehydes, genipin, multifunctional epoxy compounds and N-hydroxysuccinimide (NHS)/Polycarboxylic acids could directly react with free amino groups within different collagen chains to form crosslinking. The crosslinking induced by 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) and diphenylphosphorylazide (DPPA) begins with the activation of the carboxyl groups in collagen chains to promote the formation of amido bonds between adjacent carboxyl and amine groups of collagen. Transglutaminase (TGase) catalyzes an acyl-transfer reaction mainly involved in glutamine and lysine residues of collagen.

Aldehydes crosslink collagen via forming Schiff bases between the ε-amino groups of lysine or hydroxylysine residues and the aldehyde groups of aldehydes, as shown in Scheme 4a. Glutaraldehyde (GTA) as a typical crosslinker could remarkably enhance the thermal stability of collagen. Different states of collagen crosslinked using GTA have been investigated. 1g dermal layer, which was obtained after removing non-collagenous substances of sheepskin, was crosslinked in 100 ml 0.5% GTA solution for 1h at room temperature (pH 7.4). The Ts values of the crosslinked and the uncross-linked dermal layers



**Scheme 4** Crosslinking mechanisms of collagen with Aldehydes (a) [205], Genipin (b) [206], Epoxy compounds (c) [207], NHS-activated carboxylic acid esters (d) [208], EDC/NHS (e) [209], DPPA (f) [210] and Transglutaminase (g) [211] respectively

were 78 and 56°C respectively [212]. Calfskin collagen solutions were crosslinked using GTA at different GTA/collagen ratios (w/w) for 24h at room temperature (pH 4.0). The Td values of the uncross-linked collagen were 39.7°C and that of crosslinked solution using 1% GTA was 40.3°C. The thermal stability of the collagens and the degree of crosslinking tended to flatten out as GTA content further increased [213]. Collagen gels were prepared by incubating the collagen solutions (PBS, 100 mmol/L NaCl, pH 7.4) at 37°C for 3 h, and then cross-linked using GTA at different molar ratios of [CHO]/[NH<sub>2</sub>] where CHO was aldehyde groups of GTA and NH<sub>2</sub> was ε- amino groups of lysine or hydroxylysine residues. Td of the uncross-linked gel was 47.1°C. The Td values observably increased to 63.9°C as [CHO]/[NH<sub>2</sub>] was 0.6 and then levelled off as [CHO]/[NH<sub>2</sub>] was above 3 [214]. The improvement of thermal stability was significantly obtained when low dosage of GTA was added. The majority of reactive amino groups in collagen could form intra- and inter-molecular multipoint crosslinks with aldehyde groups of GTA. The thermal stability of collagen levels off due to the formation of single-point bonding as the dosage of GTA is over high [212]. Despite GTA-treated materials such as bioprosthesis valves and grafts have been applied to clinical, an undesirable side-effect of toxicity is an issue. Recently, dialdehyde compounds derived from natural biomass with good biodegradability and low toxicity have attracted increasing attention. The thermal stability of collagen have been demonstrated to be improved by dialdehyde compounds such as oxidized glycogen, oxidized chitosan oligosaccharide, dialdehyde cellulose, oxidized sodium alginate and dialdehyde carboxymethyl cellulose (DCMC) [215–218]. A collagen cryogel was prepared by Tan et al. [219] using dialdehyde carboxymethyl cellulose to cross-link collagen in solution at different DCMC/Col weight ratios of 0.0–0.2% with constant collagen concentration of 5 mg/ml. Td of the cryogels increased by 22°C at ratio of 0.1%, and then decreased slightly at ratio of 0.2% where insoluble complexes were found. The complex solution of collagen and the dialdehydes might be not stable enough due to large molecular weight as well as the strong electrostatic attraction between collagen and the dialdehydes. A more stable hydrogel of collagen and DCMC with a higher concentration was prepared by Yang et al. [205] using the biphasic acetic acid/1-ethyl-3-methylimidazolium acetate solvent system. The Col/DCMC solution with constant DCMC content contained different collagen concentrations of 5–25 mg/ml. The Td values exhibited a linearly upward tendency with the increase of collagen content. The gels of all the collagen concentrations were transparent and homogenous.

Genipin is an iridoid derivative. The C-atom of genipin is firstly under a nucleophilic attack from primary amine

groups of amino acids residues (Lys, Hyl, Arg), and then tertiary nitrogen of collagen embeds into the six-membered ring of genipin to replace an oxygen atom, finally a nitrogen-iridoid could be formed. Subsequently, two iridoids are linked via a radical reaction to form intra- and inter-molecular crosslinks (Scheme 4b) [206]. The report of fixing bovine pericardium using genipin came from Sung et al. [220]. The bovine pericardium was soaked in 0.625% genipin solution for 3 days at room temperature (pH 7.4). Ts of the fixed pericardium increased by about 13°C comparing with the unfixed pericardium (63.9°C). Storage stability of genipin-crosslinked and GTA-crosslinked collagen was reported by Sung et al. [221]. Porcine pericardia prepared by removing non-collagenous substances was fixed using 0.625% genipin or GTA in buffer solution (pH 7.4) at 37°C for 3 days. The samples were stored in a sterilized water after sterilizing. The Td values of GTA-fixed pericardia was 86.5°C and then began to decrease at 3 months after storage. Td of genipin-fixed pericardia was 80.5°C and kept constant until 6 months after storage. Additionally, no apparent cytotoxicity was observed for both the genipin-fixed tissue and storage solution during storage process when compared to GTA-fixed tissue.

Multifunctional epoxy compounds have strong reactivity due to three-membered cyclic ether groups. Epoxy groups of epoxy compounds could form multipoint crosslinking among collagen molecules with the ε-amino groups of lysyl residues in collagen at alkali condition (Scheme 4c) [207, 222]. The crosslinking between collagen and epoxy compounds have been demonstrated to be remarkably affected by pH [222]. 1 g sheep dermal collagen prepared by removing non-collagenous substances was immersed in 100 mL buffered solution containing 4 wt% 1,4-butanediol diglycidyl ether at the different pH values of 4–12 (20°C). At pH 7, Ts of the crosslinked collagen showed the least increase comparing with the crosslinked collagens under other pH values. Under acid condition (pH < 7), amine group content of collagen kept constant and Ts slightly increased with the decrease of the pH values. At pH = 8–10, Ts exhibited a lineal upward tendency accompanying with the evidently decrease of amine group contents. At a higher pH (>10), both Ts and amine group content were kept constant. Additionally, Ts of the crosslinked collagen in alkali condition were always much higher than that in acid condition. Shen et al. [223] pointed out the glycerol polyglycidyl ether exhibited good uniform distribution of crosslink density throughout the porcine aortic valves. Porcine aortic valves were immersed in 4% glycerol polyglycidyl ether solution for 6 days at room temperature. Hydrothermal stability of distinct layers (outer, middle, inner and entire wall as well as leaflet) in

aortic valves was almost same and was 20°C higher than that of the uncross-linked aortic valve.

The carboxyl groups of polycarboxylic acids are turned into an active ester groups by NHS under the action of EDC or 1,3-dicyclohexylcarbodiimide catalyst. Crosslinking would be formed between the active ester groups and the amino groups of collagen molecules, thus enhancing the thermal stability of collagen (Scheme 4d). Citric acid, adipic acid, gallic acid, poly ( $\gamma$ -glutamic acid) and poly ( $\alpha,\beta$ -malic acid) have been chosen into system of NHS-activated carboxylic acid esters [208, 224–230]. The properties of collagen crosslinked using NHS activated adipic acid (NHS-AA) were studied by Li and her group [208, 224–227]. Alkali-solubilized collagen of bovine hide and PSC of fish skin were dissolved in phosphate solution buffer (pH 6.3) to make collagen concentration at 0.3% (w/w). Two collagen solutions were mixed with NHS-AA of different concentrations. Td of the alkali-solubilized collagen reached to the highest (40.8°C) at NHS-AA concentration of 1.5 mM, which was 4.2°C higher than that of the uncross-linked collagen. Td of PSC increased by 13.3°C at NHS-AA concentration of 1.05 mM comparing with the native collagen [208, 224]. The decrease of amino groups in alkali-solubilized collagen due to the deamination of Asn and Gln into Asp and Glu caused less crosslinks than in PSC. The collagen self-aggregation was observed to be affected with the increase of NHS-AA. Calfskin collagen solution was crosslinked using NHS-AA of different concentrations at pH 7.2 and then heated from 4°C to 37, 39 and 41°C within 1 min to form fibrils. Concentrations of NHS-AA were converted to molar ratios of [NHS-AA]/[NH<sub>2</sub>] where [NHS-AA] was active ester groups and [NH<sub>2</sub>] was  $\epsilon$ -amino of lysine and hydroxylysine residues. The turbidity and the rate of fibril formation decreased as the NHS-AA increased, especially at a [NHS-AA]/[NH<sub>2</sub>] > 0.6/1. The self-aggregation was still carried out at a [NHS-AA]/[NH<sub>2</sub>] ratio < 0.3/1 when temperature was below 39°C [225]. The properties of collagen gels (fish skin) prepared by incubating the complex solutions (pH 7.4) of collagen and NHS-AA at 30°C for 5h were further studied. The gels became transparent as [NHS-AA]/[NH<sub>2</sub>] was above 0.2. The degree of crosslink observably increased with the increase of NHS-AA and then leveled off at a [NHS-AA]/[NH<sub>2</sub>] ratio > 0.8. Td of the gel increased by 7.9°C at ratio of 1.6 comparing with the uncross-linked gel [226]. Different thermal stability of collagen gels was revealed by changing the order of adding NHS-AA. The collagen gels (calfskin) were crosslinked using NHS-AA of the same dosage before the self-aggregation (CBF), simultaneously with and after the self-aggregation (CSF and CAF) respectively. The thermal stability of the gels exhibited in order of CAF > CSF > CBF. The appearance of the gels was non-

transparent for CAF and became transparent for CSF and CBF at a [NHS-AA]/[NH<sub>2</sub>] ratio > 0.6/1 [227]. Additionally, the isoelectric point of crosslinked collagen could be decreased via introducing extra carboxyl groups by NHS-activated carboxylic acid esters to made it possible to dissolve in the neutral water [228–230].

As “zero-length” crosslinker, EDC and DPPA promote the formation of amido bond between adjacent carboxylic and amino groups of collagen and don't stay in the systems [209, 210]. The activated mechanisms of carboxylic groups of Glu and Asp residues induced by EDC and DPPA are different. EDC converts carboxyl groups of collagen into active O-acylisourea groups, subsequently crosslinking takes place after nucleophilic attacking O-acylisourea groups by free amine groups of lysine or hydroxylysine residues. Usually, during the EDC crosslinking procedure, NHS is added as a catalyst to significantly enhance the crosslinking efficiency owing to the formation of more stable active intermediates (Scheme 4e). The thermal stability of the collagen crosslinked using EDC and EDC/NHS respectively was compared in Old Damink's study [231]. 1g sheep dermal layers obtained after removing non-collagenous substances were immersed into 100 ml aqueous solution containing 1.15g EDC or 1.15g EDC and 0.69g NHS at room temperature for 4h. The pH value was kept at 5.5 during the reaction. Ts of the dermal layer treated with EDC increased by 17°C and that treated by EDC/NHS increased by 30°C comparing with the native dermal layer (56°C). DPPA converts carboxyl groups into acylazide groups which further react with the amino groups of adjacent collagen chains (Scheme 4f). The thermal stability of the collagen treated with DPPA was significantly affected by the solvent used for DPPA [210]. Herbage and his group immersed 10 mg dry pericardial samples into 5 ml 1% DPPA solution in different solvents at 4°C for 24 h. Td of the untreated sample was 67.3°C. The Td values of the samples treated with DPPA in dimethylformamide, acetone and dichloromethane were 81.9, 72.4 and 67.4°C respectively. The thermal stability of the collagen treated with DPPA (according to the above method) and GTA respectively was further compared. Collagen gel was obtained after complete swelling of calf dermis powder in acetic acid. Collagen sponge was obtained by lyophilizing the 0.7% collagen gel and film were prepared by drying 1.5% gel at room temperature for 3 days. The sponge and film were crosslinked using 1% GTA at 20°C and pH 6.2 for 24 h. The thermal stability of the DPPA-treated sponge and film increased by 20°C comparing with the untreated sponge and film. The Td values of GTA-treated sponge and film were 27 and 22°C higher than that of the uncross-linked sample, respectively [232]. At the optimal condition, the thermal stability of collagen could be significantly improved by DPPA.

TGase-mediated crosslinking is a nontoxic method for stabilizing collagen and collagenous materials. As an enzyme in various organisms, TGases are involved in essential biological processes [211]. The  $\gamma$ -carboxamide groups of glutamine residues catalyzed by TGases are acyl donor and the  $\epsilon$ -amino groups of lysine residues or primary amino groups act as acyl acceptor. Consequently, the intra- or inter-molecular crosslinks can be achieved via the formation of  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds (Scheme 4g) [233]. The thermal stability of the collagen treated with TGase was affected by the different states of collagen. An evident improvement of thermal stability of the TGase-treated collagen solution was reported by Chen et al. [234]. Porcine skin collagen solution (10 mg/ml) was added with TGase (60 U/g collagen) or without and then incubated at 25°C and pH 4 for 12 h. The sponges were obtained after lyophilizing the solutions. The Td values of the untreated and TGase-treated sponges were 114 and 177°C respectively. TGase showed no contribution to thermal stability of collagen fibers in Cheng's study [235]. 10g/kg collagen fiber suspensions prepared by removing non-collagenous substances from limed bovine skin were added with TGase (20 U/g collagen) and then reacted at room temperature for 4h. Td of the TGase-treated collagen fibers was 82.95°C which was same as the native fibers (82.58°C).

The thermal stability of collagen or collagenous materials could be significantly improved by crosslinking. Usually, the denaturation temperature of collagen as insoluble forms such as fibers, films and sponges are always much higher than that in solution. Therefore, physical crosslinking, which introduce strong energy into collagen, is more suitable for modification of dried collagen matrixes. The degree of chemical crosslinking would be affected by different states of collagen. The collagen molecules in solution are more scattered, which is not conducive to the formation of intermolecular crosslinks. For leather industry, the hydrothermal stability of leather is very important. The desirable properties of leather could be easily obtained by the use of chromium and GTA in spite of the toxic issue. Besides of thermal stability, the suitable physicochemical property and acceptable biocompatibility are also taken major concern when collagen is applied in biomedicine fields. The selection and combination of crosslinkers and crosslinking methods are necessary during practical processing.

## 7 Conclusion

Intact triple helical structure endows collagen excellent characteristics which is the premise for diversified applications. The native structure of collagen would collapse during heating process. The thermal stability is a macroscopic expression of collagen structure stability. The

studies of thermal denaturation mechanism have been developed from traditional statistics to model simulation which could more accurately reveal the thermal denaturation behavior of collagen. Usually, collagen is extracted into aqueous solution for direct use or to be further manufactured into gel, fiber, membrane, sponge, scaffold and so on according to various applications. The stability of triple helix would be easily affected by the use of solvent and physical assisted means. The monitoring of thermal stability is necessary for helping to adjust the processing technology. Modification of collagen is carried out to promote thermal stability, mechanical property and resistance to enzymatic degradation. The demand of thermal stability varies with the different uses of collagenous materials. Hydrothermal stability is the most important standard for leather. However, for biomedical materials, good biocompatibility and low toxicity are also taken major concern. The choice of reagents and assisted means during processing and the use of modified methods should adapt to the demand of practical applications.

### Abbreviations

ASC: Acid-soluble collagen; PSC: Pepsin-soluble collagen; Col: Collagen; AA: Acetic acid; Gly: Glycerol; RTT: Rat tail tendon; ILs: Ionic liquids; AMS: Diethyl methyl ammonium methane sulfonate; BCS: Bis-choline sulphate; IDP: 1-butyl-3-methyl imidazolium dimethyl phosphate; cDHP: Choline dihydrogen; [EMIM][Cl]: 1-ethyl-methylimidazolium chloride; [EMIM][Br]: 1-ethyl-methylimidazolium bromide; [EMIM][Ac]: 1-ethyl-3-methylimidazolium acetate; [EMIM][BF<sub>4</sub>]: 1-ethylmethylimidazolium tetrafluoroborate; [EMIM][N(CN)<sub>2</sub>]: 1-ethyl-methylimidazolium dicyanamide; [BMIM][Cl]: 1-butyl-3-methylimidazolium chloride; [BMIM][BF<sub>4</sub>]: 1-butyl-3-methylimidazolium tetrafluoroborate; AA/[EMIM][Ac]: Acetic acid/1-ethyl-3-methylimidazolium acetate; CS: Chondroitin sulfate; C4S: Chondroitin 4-sulfate; HA: Hyaluronic acid; HPMC: Hydroxypropyl methylcellulose; CMC: Carboxymethyl cellulose; CH: Chitosan; PVA: Poly-vinyl-alcohol; PVP: Poly-vinyl-pyrrolidone; PEG: Poly-ethylene-glycol; PLGA: Poly-D, L-lactide-co-glycolide; HFP: 1,1,1,3,3,3-hexafluoro-2-propanol; GTA: Glutaraldehyde; DCMC: Dialdehyde carboxymethyl cellulose; NHS: N-hydroxysuccinimide; EDC: 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; NHS-AA: NHS activated adipic acid; DPPA: Diphenylphosphorylazide; TGase: Transglutaminase;  $\epsilon$ : Specific dielectric constant; DHT: Dehydrathermal; UV: Ultraviolet;  $[\eta]$  *exp* *m*: Specific viscosity;  $[\eta]$  *i m*: Ideal intrinsic viscosity; PBS: Phosphate buffered saline; CD: Circular dichroism measurements; DSC: Differential scanning calorimetry; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; FTIR: Fourier transform-infrared

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### Authors' contributions

GYL made substantial contributions to conception and design of this review, and critical revised the manuscript. XXZ wrote and revised the manuscript. SCX participated in the design of this review. LRS revised the draft from the language and logicity aspects. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets analyzed during the review are available in the references which have been specified in the article.

**Competing interests**

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