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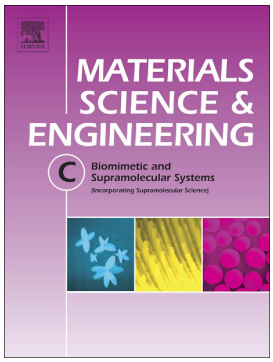

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Influence of telopeptides on the structural and physical properties of polymeric and monomeric acid-soluble type I collagen

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Abstract

Currently two factors hinder the use of collagen as building block of regenerative devices: the limited mechanical strength in aqueous environment, and potential antigenicity. Polymeric collagen is naturally found in the cross-linked state and is mechanically tougher than the monomeric, acid-soluble collagen ex vivo. The antigenicity of collagen, on the other hand, is mainly ascribed to inter-species variations in amino acid sequences of the non-helical terminal telopeptides. These telopeptides can be removed through enzymatic treatment to produce atelocollagen, although the effect of this cleavage on triple helix organization, amino acidic composition and thermal properties is often disregarded. Here, we compare the structural, chemical and physical properties of polymeric and monomeric type I collagen with and without telopeptides, in an effort to elucidate the influence of either mature covalent crosslinks or telopeptides. Circular dichroism (CD) was used to examine the triple helical conformation and quantify the denaturation temperature (T_d) of both monomeric collagen (36.5 ^oC) and monomeric atelocollagen (35.5 ^oC). CD measurements were combined with differential scanning calorimetry (DSC) in order to gain insight into the triple helix-to-coil thermal transition and shrinkage temperature (T_s) of polymeric atelo collagen (44.8 ^oC), polymeric collagen (62.7 ^oC), monomeric atelo collagen (51.4 ^oC) and monomeric collagen (66.5 ^oC). Structural and thermal analysis was combined with high pressure liquid chromatography (HPLC) to determine the content of specific collagen amino acidic residues used as markers for the presence of telopeptides and mature crosslinks. Hydroxylamine was used as the marker for polymeric collagen, and had a total content of 9.66% for both polymeric and polymeric atelo collagen; tyrosine was used as the marker for telopeptide cleavage, was expressed as 0.526% of the content of polymeric collagen and the partially-reduced content of 0.39% for atelocollagen.

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Keywords: polymeric collagen; atelocollagen; telopeptides

1. Introduction

The biocompatibility and versatility of collagen derived products for medical applications has long been recognized and reflected in its wide scale research [1]. As a scaffold material, it benefits from enzymatic degradability, affinity for biomolecules and exhibits organizational and macromolecular properties similar to the natural extracellular matrix (ECM) [2]. Its wide application includes sutures, tissue replacement and regeneration, cosmetic surgery, dental membranes and skin regeneration templates [3]. Collagen has a unique triple helix structure made of three left-handed polypeptide (α-chains) chains held together by hydrogen bonds between the peptide bond (NH) of a glycine residue with the peptide carbonyl (C=O) of the adjacent polypeptide [4]. Each chain follows the amino acid motif, -Gly-X-Y-, where X and Y are often proline and hydroxyproline [5]. The steric repulsion between proline and hydroxyproline provide the C-N fixed angle which allows the α-chains to fold into three tight, left-handed coils. These are able to stack together to form the right-handed triple helix due to the presence of glycine every third position. The small glycine residues can be orderly packed inside the crowded center of the triple helix, while the other amino acids are on the outside, their presence inside the coil provides an available hydrogen atom to form the hydrogen bonds and stabilize the triple helical structure [6]. The short segments of the C- and N- termini of the polypeptides (the telopeptides), do not possess the repeating Gly-X-Y motif and are therefore non-helical [7, 8]. Instead, the end telopeptides possess an uncommon amino acid hydroxylysine which is important for the formation and stabilization of collagen fibrils due to the action of lysyl oxidase which catalyses the covalent aldol reaction between the lysine or hydroxylysine residues in the N- and C- teleopeptides of adjacent molecules, thus bonding two molecules head-to-tail along the fibril [4, 6, 9]. Covalent cross-link also occurs within the triple helical sequence by means of lysine hydroxylation and hydroxylysine glycosylation [10]. These intermolecular cross-links account for almost all bonds the younger tissue.

These natural cross-links are chemically rearranged with age to form acid-stable aldminine cross-links, which provide the increased mechanical strength of mature tissue [11, 12].

Collagen fibrils possess high tensile strength with a diameter between 50 – 200 nm, and when packed side-by side in tissues such as tendon, form collagen fibers [4, 13]. Covalent cross-links of collagen fibrils can be broken down ex vivo via incubation of tissue in weak acidic solutions such as 17.4 mmol acetic acid, so that monomeric collagen can be isolated as building block for biomaterial design.

The use of biological material for medical applications requires a distinction between immunogenicity and antigenicity. Immunogenicity is about triggering an immune response, or the degree to which it invokes a response, whilst antigenicity can be determined by macromolecular features of an antigen molecule such as three-dimensional (3D) conformation and amino acid sequence [14].

Collagen, as an animal-derived biomaterial has always raised concerns regarding its potential to evoke immune responses [7, 15-17]. However, the interpretation of immunochemical reactions to collagen-containing implants is often complicated by the presence of cell remnants, or chemicals from extraction or cross-linking treatments [18-20].

1.1. Atelocollagen

The collagen molecule can be divided into three domains: the terminal amino (N-) telopeptide, the triple helix, and the terminal carboxy (C-) telopeptide [21]. Treatment of collagen with proteolytic enzymes (e.g. pepsin or ficin) can cleave the terminal telopeptides to produce atelocollagen with an intact triple helical conformation [5].

It has been claimed that the majority of collagen antigenicity is attributed to the terminal telopeptides, however, the biological effects of atelocollagen are not yet fully understood [14]. The antigen determinants for collagen can be classified in three categories: helical recognition by antibodies dependent on 3D conformation

located within the triple helical portion of native collagen, amino acid sequence and terminal located in the non- helical telopeptides of the molecule. It had been eluded that collagen devoid of terminal telopeptides can eliminate its immunogenicity [14, 22]. The removal of telopeptides often results in an amorphous arrangement of collagen molecules and a loss of the collagen fibril pattern in the reconstituted product, due to the roles of the C- and N-terminus telopeptides in cross-linking and fibril formation [14, 23]. The induced positively charged surface of the atelo collagen can significantly increase its solubility and therefore processabilty of the collagen as a biomaterial.

1.2. Polymeric collagen

All collagenous tissues contain a fraction of soluble monomeric collagen which is extractable in weak acidic solutions. In mature tissues, such as tendons, the bulk of the collagen consists of insoluble, highly cross-linked polymerized fibers of type I collagen (polymeric collagen) with a smaller amount as acid-soluble monomeric collagen (<10 %) [12, 24]. The natural cross-links are chemically rearranged with age to form acid-stable aldminine cross-links, which provide increased mechanical strength of the tissue [11, 12].

The aim of the present study was to assess the influence of telopeptide removal on the structural and physical properties of polymeric and monomeric type I collagen.

2. Methods

2.1. Materials

Monomeric atelocollagen (Collagen Solutions, UK), polymeric collagen and polymeric atelocollagen (Southern Lights Biomaterials, New Zealand).

2.2. Chemical and structural characterisation

Circular dichroism (CD) spectra of collagen samples were acquired (ChirascanCD spectrometer, Applied Photophysics Ltd) using 0.2 mg·ml^{-1} solutions in HCl (10 mM). A homogenizer was used to dissolve polymeric and atelo polymeric collagen. Sample solutions were collected in quartz cells of 1.0 mm path length, whereby CD spectra were obtained with 4.3 nm band width and 20 nm.min^{-1} scanning speed. A spectrum of 10 mM HCl solution was subtracted from each sample spectrum.

$$\theta_{mrw,\lambda} = \frac{MRW\ x\ \theta_{\lambda}}{10\ x\ d\ x\ c} \quad (1)$$

Where θ_{λ} is the observed molar ellipticity (degrees) at wavelength λ, d is the pathlength (1 cm) and c is the concentration (0.2 mg·ml^{-1}) [25].

A temperature ramp was conducted from 20 to 60 °C with 20 °C/hour heating rate with ellipticity measurements at 221 nm fixed wavelength. The 221 nm coincides with the positive band associated with the collagen triple helix and its destruction will be related to a lower value of ellipticity. The denaturation temperature (T_d) was determined as the mid-point of thermal transition.

Differential Scanning Calorimetry (DSC) was used in order to investigate the thermal denaturation (T_m) of collagen samples (TA Instruments Thermal Analysis 2000 System and 910 Differential Scanning Calorimeter cell base). DSC temperature scans were conducted with 10-200 °C temperature range and 10 °C.min^{-1} heating rate. 5-10 mg sample weight was applied in each measurement and three scans were used for each sample formulation. The DSC cell was calibrated using indium with 20 °C·min^{-1} heating rate under nitrogen atmosphere.

High pressure liquid chromatography (HPLC) was used to investigate the amino acid occurrence in collagen samples (Dionex Ultimate 3000 HPLC, Dionex Softron GmbH, Germany). For acid stable amino

acids, hydrolysis was performed in 6 M HCl solution for 24 hours at 110 ^{o}C in an evacuated sealed tube followed by fluorescence detection. Results were calculated as residues per 1000 residues.

2.3. Physical characterization

Scanning electron microscopy (SEM) was used for microscopic analysis of collagen by gold-coating the samples in order to examine the internal material architecture. Samples were mounted onto 10 mm stubs and electron micrographs captured (FEI Quanta 600) via backscattered electron detection at 10 kV and 12 – 13 mm working distance.

3. Results and discussion

3.1. Chemical and structural characterization

CD is defined as the unequal absorption of left-handed and right-handed circularly polarized light. When the chromophores of the amides of the polypeptide backbone of proteins are aligned in arrays, their optical transitions are shifted or split into multiple transitions [26]. The CD spectra of proteins are dependent on their conformation, so CD is a useful tool to estimate the structure of unknown proteins and monitor protein conformational changes due to environmental factors or irreversible denaturation [26]. Far-UV CD spectra of monomeric type I collagen displayed a positive band at 221 nm and a negative band at 198 nm, characteristic of the triple helical conformation which decreases during denaturation (figure 1.a).

The magnitude ratio of the positive to negative band (RPN) in monomeric type I collagen spectra was found to be 0.094, comparable to the literature value of native collagen, RPN: 0.117 [27].

The monomeric atelocollagen had an RPN value of 0.189. The positive band at 221 nm at similar molar ellipticity to the 221 nm-band of monomeric collagen suggests a preserved triple helical conformation, despite the removal of the telopetides and the covalent inter-strand cross-links they provided.

Polymeric type I collagen displayed a wide positive band centered at 214 nm, characteristic of random coils and no negative band. The change in the absorption wavelengths is evidence for alterations in the secondary structure, in terms of electronic transitions in the chain backbone or in the helically arrayed side groups of collagen [28]. The spectra could imply that the natural cross-links found in the mechanically stronger polymeric collagen, hinder the coiled supramolecular assembly characteristic of monomeric collagen.

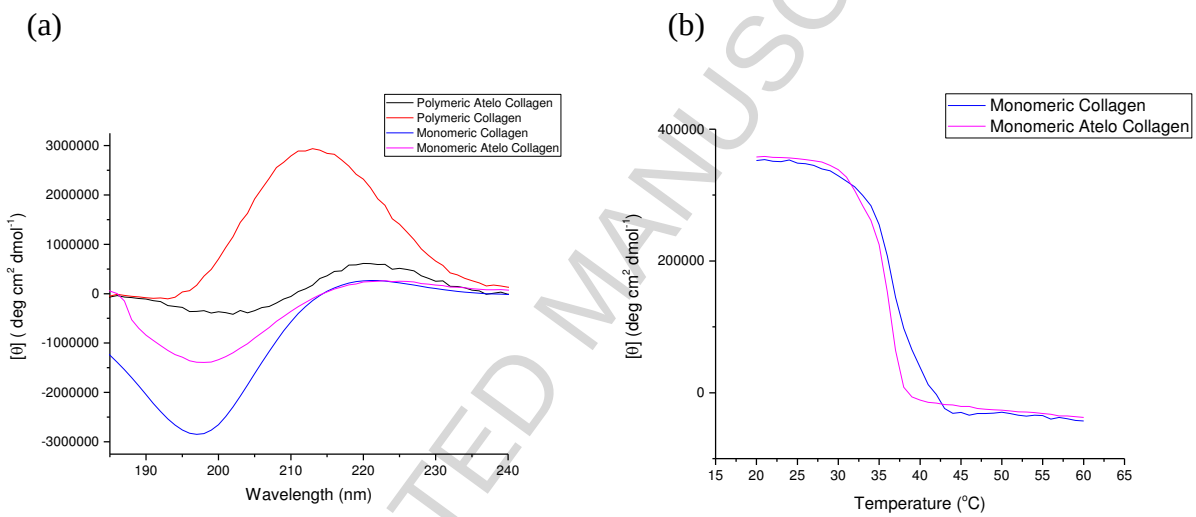

Fig. 1. (a) CD graph polymeric atelo, polymeric, monomeric atelo and monomeric type I collagen; (b) temperature ramp graph to show denaturation of monomeric collagen and monomeric atelo collagen

The spectra of polymeric atelo collagen (RPN: 1.21; the native monomeric collagen RPN: 0.117) bore no resemblance to the broad positive band displayed for polymeric collagen, and instead showed a positive band (221 nm) similar to monomeric atelocollagen. This could suggest that the enzyme-catalyzed procedure to cleave the telopeptides, also resulted in the destruction of the natural cross-links that differentiate polymeric

collagen from monomeric collagen. However, the difference between the molar ellipticity of the positive and negative peak for polymeric atelocollagen suggest some denaturation of the triple helical structure.

A temperature ramp between 20 and 60 ^{o}C was used to follow the denaturation of collagen triple helices to random coils. This could not be performed on polymeric collagen due to the lack of the characteristic 221 nm band maximum. Instead, it was used as a tool to examine the influence of telopeptides on the denaturation temperature of monomeric collagen (figure 1.b).

Denaturation temperature (T_d) was measured at half the initial molar ellipticity of the characteristic positive band (221 nm). For monomeric collagen, T_d was 36.5 ^{o}C and monomeric atelo collagen, T_d was 35.5 ^{o}C.

These differing values could be due to the subdomain of collagen, and the roles that the C- and N-telopeptides provide for intermolecular covalent cross-links which help to stabilize the collagen triple helix; without these additional interactions, the atelocollagen triple helices denature at a lower temperature.

DSC was used to determine shrinkage, indicated by T_s, related to the thermal transitions of the collagens on heating and was employed in this study to investigate the effect telopeptide cleavage has on the thermal

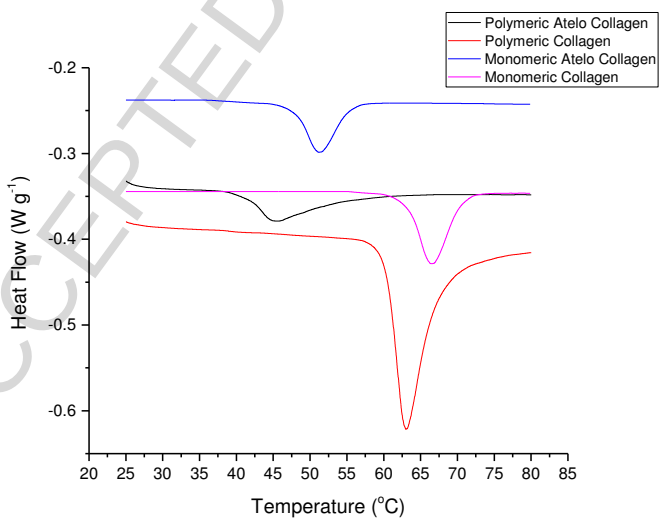

Fig. 2. DSC data for polymeric atelo, polymeric, monomeric atelo and monomeric type I collagen

properties [29] (figure 2). Higher shrinkage temperatures (peak maximum) are typically indicative of a higher degree of intermolecular interactions between the collagen molecules. Telopeptide cleavage results in a smaller

shrinkage temperature for both polymeric and monomeric collagen (table 1). This result can be contributed to the covalent aldol cross-links provided by the C- and N- terminus to the adjacent molecule.

Table 1 DSC data from polymeric atelo, polymeric, monomeric atelo and monomeric type I collagen

Collagen Type I	Average Enthalpy ($W \cdot g^{-1}$)	Average Peak Maximum (^oC)
Polymeric Atelo	0.378	44.83
Polymeric	0.621	62.69
Monomeric Atelo	0.320	51.42
Monomeric	0.432	66.53

High pressure liquid chromatography (HPLC) was used to determine the amino acid content (residues per 1000 residues) in polymeric atelo and polymeric type I collagen (table 2). Hydroxyproline is formed intracellularly from the post-translational hydroxylation of proline and constitutes 10–14% of the total amino acid content of mature collagen [30]. The hydroxyproline content for both collagenous proteins was 9.66% of the total amino acid content (96.6 residues per 1000 residues) for polymeric collagen confirming to the literature value for mature (polymeric) collagen. Tyrosine is only present in the telopeptides of the collagen molecule and can be used as a measure of telopeptide removal [28]. Existing normally as 0.5% of the total amino acid content, a total content below 0.2% (2 residues per 1000 residues) was found to serve as a suitable

measure for sufficient removal of telopeptides [31, 32]. The amino acid sequence of the telopeptides shown below for bovine type I collagen [33]:

The free α (1) N-terminal telopeptide conformation:

α1 GLU-LE-SER-TYR-GLY-TYR-ASP-GLU-LYS-SER-THR-GLY-ILE-SER-VAL-PRO

The free α (1) C-terminal telopeptide conformation:

α1 SER-GLY-GLY-TYR-ASP-LEW-SER-PHE-LEU-PRO-GLN-PR-PPRO-GLN-GLX-LYS-ALA-HIS-ASP-GLY-GLY-ARG-TYR-TYR-ARG-ALA

Polymeric collagen had a tyrosine total content of 0.53% and polymeric atelocollagen had a total content of 0.39%. This result is higher than the literature value of atelocollagen, however, the previous data from CD and DSC showed a definite difference between polymeric atelo and polymeric collagen. Instead, an explanation for the higher tyrosine occurrence could be as a result of telopeptide docking, whereby the free terminal structure docks onto the triple-helix chain as a staggered structure, so the tyrosine would still be accounted for in HPLC [34].

Table 2 HPLC data from polymeric atelo and polymeric collagen displayed as residues per 1000 residues

Amino Acid	Atelo Polymeric Collagen (residues per 1000 residues)	Polymeric Collagen (residues per 1000 residues)
Aspartic Acid	46.2	47.26
Threonine	16.2	16.36
Serine	34.1	33.19
Glutamic Acid	73.7	72.26
Proline	123	122.54

Glycine	337	334.00
Alanine	108	108.77
Valine	22.9	23.54
Methionine	6.31	6.61
Isoleucine	12.9	13.68
Leucine	25.23	26.26
Tyrosine	**3.90**	**5.26**
Phenylalanine	13.6	13.81
Histidine	5.70	5.85
Lysine	22.2	21.44
Arginine	52.0	52.57
Hydroxyproline	**96.6**	**96.6**

3.2. Physical characterization

SEM images were taken at varying magnifications to examine the internal material architecture (figure 3.). Different morphologies are observed at 2000x and 8000x magnification after telopeptide cleavage.

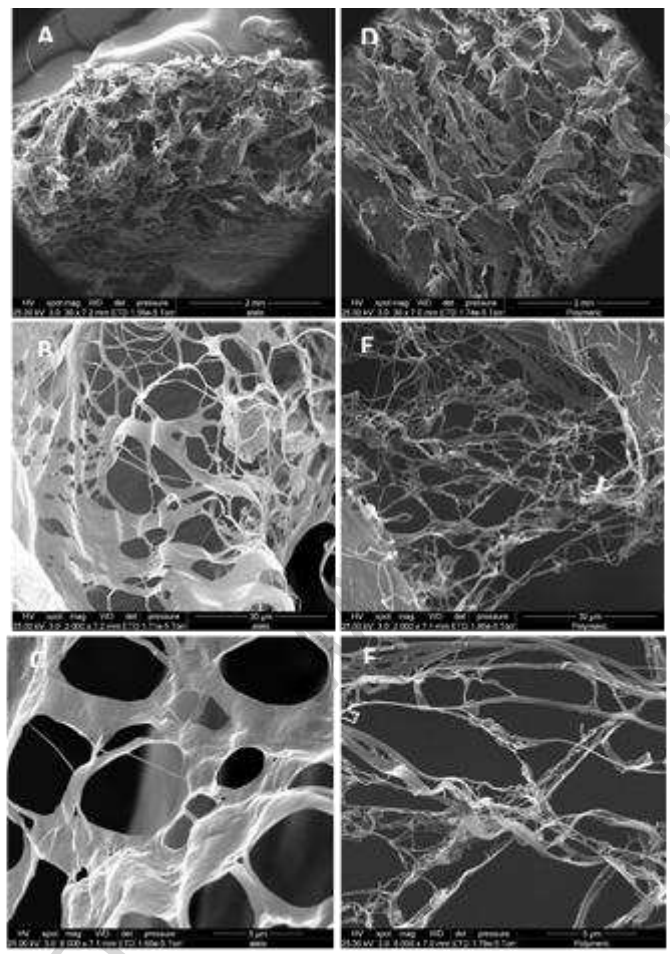

Fig. 3. SEM images of polymeric atelo collagen (A-C) and polymeric collagen (D-F). Magnifications: A&D: 30×; B&E: 2000×; C&F: 8000×.

4. Conclusions

This study showed that monomeric atelocollagen displayed the characteristic positive band at 221 nm on the CD spectrum associated with the triple helical conformation of collagen, which implies that despite the

covalent aldol cross-links provided by the telopeptides, their cleavage doesn't disrupt the natural collagen structure. It was found that polymeric collagen didn't display the characteristic positive peak at 221 nm, thereby implying that the natural cross-links associated with mature collagen disrupt the native triple helical structure. The influence of telopeptides was noted in the DSC results with decreased T_S values after telopeptide cleavage for both monomeric and polymeric collagen, likely due to the reduced intermolecular aldol covalent cross-links attributed to the telopeptides. Telopeptide removal was confirmed using HPLC (decreased tyrosine content) with potential telopeptide docking.

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