Self-Assembly Study of Type I Collagen Extracted from Male Wistar Hannover Rat Tail Tendons

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Abstract

**Background:** Collagen is the main structural protein in the extracellular matrix in the numerous connective tissues in the body. As the main component of connective tissue, it is the most abundant protein in mammals.

**Method:** Collagen Type I from Wistar Hannover rat tail was obtained and it allowed to describe the thermodynamic profile during self-assembly by isothermal titration calorimetry (Nano ITC) as a novel technique.

**Results:** The enthalpy of self-assembly of the 0.01035 moles of collagen was 33.89 mJ; relevant data in tissue engineering and 3D collagen fibrils bio-impressions organs, with a periodicity of 65 nm were obtained and characterized.

**Conclusions:** Calorimetric analysis shows higher energy release event at the isoelectric point of the protein, suggesting an overall exothermic process due to self-assembly of the collagen and an endothermic process due to aggregation of denatured collagen.

Introduction

Collagen is one of the most important structural proteins, accounting for up to one quarter of protein biomass in mammals [1]. The interest in this protein has risen in the last years due to the relevance of its properties related to important biological functions such as cell attachment and the patterning or structuring of tissues [2]. Collagen can be used not only as a scaffolding matrix for tissue engineering applications, but also as a coating of non-biological surfaces to improve and secure their biocompatibility [3].

According to the shape of the fibrils and their arrangements, 25 subtypes of collagen have been identified [4], Type I, II and III collagen are characterized by its fibrillary nature, whereas Type IV collagen is amorphous [5]. The peptide chains that compose Type I collagen present a helical domain with 338 repetitions of the short motif (X-Y- Gly), where X and Y usually correspond to proline and hydroxiproline, which are displayed on the N terminal domains and non-helical and C terminal domains [4, 6].

Importantly, there is a great demand of Type I collagen, since it is the principal proteinaceous constituent of tendons, skin, ligaments and other bone tissues. The rat tail tendon, on the other hand, is a good source of collagen because the aldimine intermolecular crosslinks can be opened easily at predominantly acidic pH, and offer good thermal and mechanical properties [7]. This is one of the main reasons why in this work, Type I collagen extracted from Wistar Hannover rats and it was used as a raw material for the preparation of gels and dry films. The collagen was extracted, reviewing the effectiveness of the proposed purification method, and characterized by its physical and mechanical properties.
The self-assembly collagen process has been previously explored in earlier studies [3]. For instance, it has been shown that the \textit{in vitro} fibrillogenesis of triple helical collagen can be controlled through pH adjustments of acid solutions which are brought to a neutral pH [4], or when the concentration of electrolytes in solution are regulated during the process of fibrillary self-assembly [3]. This research aims to understand the collagen self-assembly process from a thermodynamic and morphological point of view. This feature is transcendental to provide relevant information on the thermodynamics of the self-assembly process of this protein, and for elucidating its interactions and its role in cellular processes, especially useful for tissue engineering applications. For the characterization of the collagen, several techniques were used such as Circular Dichroism (CD), Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Amplitude Modulation Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM) and Dynamical Mechanical Thermal Analysis (DMTA).

\section*{Material And Methods}

\textbf{Type I collagen extraction.} Tendons were extracted from Wistar Hannover male specimens, provided by the Laboratorio de Ensayos Biológicos (LEBI) of the Universidad de Costa Rica (UCR). Approximately 1 g of tendon was solubilized on 200 ml of 3\% acetic acid solution with agitation, for 24 h at 4 °C. The solution was filtered at room temperature with gauzes and centrifuged at 4500 rpm for 30 min. The supernatant was lyophilized at 1.3 mbar, -20 °C, for 240 h.

\textbf{Collagen type I gelation.} Lyophilized collagen was dissolved in 3\% v/v acetic acid solution while bringing the pH 7.47 by adding dropwise a 1 N solution of sodium hydroxide (NaOH). The solution was incubated overnight at 4 °C for gelation. The gel was centrifuged and washed three times with Type I ultrapure water and was dialyzed with a Spectra/Por 3 membrane (32 mm diameter, 3.2 ml/cm volume) shaking overnight at 4 °C during 4 d. Water was changed every 2 h. For non-dialyzed collagen, these steps were omitted. Whenever dry films of collagen were needed, the gel was dried on an oven at 45 °C during 4 d.

\textbf{Collagen characterization.} Circular dichroism (CD) spectra of the collagen was obtained at 20 °C in a 10 mm path length cuvette over 190–250 nm. A CD spectrometer (J-815 Jasco Corporation, Japan) was used with signal averaging over 3 s per 0.5 nm interval at a concentration of 0.2 mM. Two repeat scans were obtained and the baseline spectrum was subtracted from the average. Subtraction of the blank was performed for smoothing the spectrum obtained. CD data was expressed as molar ellipticity values [8].

\textbf{Fourier transformed infrared spectroscopy (FTIR).} A Nicolet 6700 spectrophotometer was used, encompassing 4000 to 400 cm$^{-1}$ wave numbers with a standard resolution of 0.09 cm$^{-1}$ and using a scanning speed of 32 spectrum/s.

\textbf{Differential scanning calorimetry (DSC).} Thermal properties were performed on a TA Q200 machine, using a temperature ramp of 10 °C/min with scans over the range of 20–200 °C. Aluminum containers were used and sample mass was 5 mg.
Amplitude modulated atomic force microscopy (AFM). Dry films of collagen were directly deposited on the sample holders. AFM images were obtained with an MFP-3D Classic system (Asylum Research, CA), using tapping mode at room temperature.

Scanning electron microscopy (SEM). Samples were deposited on the sample holders, as films. SEM images were obtained from a Hitachi TM-3000 tabletop microscope operating at 5 kV, using a charge-up reduction (low vacuum) mode.

Dynamic Mechanical Thermal Analysis (DMTA). The thermo-mechanical properties of collagen were performed at room temperature (25 °C), using a Q500 (TA Instruments) equipment, with a testing strain of 10%, a 65 mm gap load, a 4.5 mm clamp face and a 16.66 µm/s gap speed. Tensile specimen dimensions were 4.5–5 mm width, 27–29 mm length and 1–1.6 mm thickness.

Isothermal titration calorimetry (ITC). ITC study was performed using a TA Nano ITC machine. Solutions of collagen in 0.33% v/v acetic acid were prepared to a final concentration of 5 mg/ml. After agitating the solution for 2 hours, the solution was measured to a volume of 25 ml with acetic acid. Then, a NaOH solution was prepared with a final concentration of 4.6 µM. All solutions were degassed by shaking them at 1300 rpm at 30 °C for 30 min. The titration was carried out by a systematic injection (12 µl) of the NaOH solution into the sample cell containing collagen or gelatin and acetic acid solution, in a 5 mg/ml concentration, under continuous stirring (300 rpm).

Results

Structural characterization of collagen films.

Figure 1. Circular dichroism (CD) spectra of collagen extracted from rat tail tendon.

Figure 2. Fourier transformed infrared spectroscopy (FTIR) spectra of extracted collagen in different conditions: freeze-dried and self-assembled: dialyzed and undialyzed.

Also, changes in Differential scanning calorimetry (DSC) thermogram of rat tail tendon collagen type I in different conditions: freeze-dried and self-assembled: dialyzed and undialyzed are shown in Fig. 3.

Figure 5. Atomic force microscopy (AFM) self-assembled collagen images in the mode of (a) Height 10 µm, (b) Amplitude 10 µm, (c) Height 2.5 µm, (d) Phase 2.5 µm.

Figure 7. Heat aggregation of extracted collagen by isotherm titration calorimetry (Nano ITC) sensed from the injection of: a) the base solution over the acid, b) the acid solution over collagen, c) the base solution over the acid collagen solution, d) the base solution over the collagen gelatin.

Figure 8. Gelation thermodynamics of extracted collagen by isotherm titration calorimetry (Nano ITC) sensed from the injection of: a) the base solution over the acid and salt, b) the collagen solution over acid
solution, c) the base solution over the acid collagen solution, d) the base solution over the collagen gelatin.

Finally, Fig. 8 shows the gelation thermodynamics of extracted collagen by isotherm titration calorimetry (Nano ITC) sensed from the injection of (a) the base solution over the acid and salt, (b) the collagen solution over acid solution, (c) the base solution over the acid collagen solution and (d) the base solution over the collagen gelatin.

**Discussion**

In vertebrates, the triple helical structure (TH) is a major structural pattern observed in collagen [9], consisting in three supercoloidal ppII conformed by amino acid residues arranged in repeated sequences X-Y-Gly, where X, Y are very frequently L-proline (Pro) and 4R-hydroxy-L-proline (4R-Hyp), respectively. Hydroxylation of Pro in Y position is essential to the fold and to the stabilization of TH [10]. In each collagen chain containing near 1000 residues, more than 100 residues are 4r-Hyp [9]. CD analysis gave information about the secondary structure of extracted collagen in solution [11, 12]. CD measurements (Fig. 1) showed the characteristic curve for the collagen triple helical structure [13, 14] with a minimum strong negative band close to 200 nm and a weak positive band close to 220 nm, indicating the presence of random coil structures, consistent with the spectrum of polyproline II Helices (ppII) [15, 16].

To evaluate the purification process of the extracted collagen and to verify significant changes on the molecular structure of collagen during self-assembly, we used FT-IR.

The three IR spectra from Fig. 2 represent the collagen in three different conditions: freeze-dried, dialyzed and undialyzed. There are typical signals from the composition of collagen type I, such the signal for amide A around 3400 cm$^{-1}$, in which there's a NH stretching along with the hydrogen bonds, and the amide B signal, visible as a peak near to 2900 cm$^{-1}$, in which there is a symmetric stretching for CH$_2$ [17].

The peaks for the Amides I, II and III matched the values described for other collagens. Amide I shows a peak near 1640–1670 cm$^{-1}$[18] owing to the stretching of the double bond between C-N and C-O [19]. The C-O group indeed generates hydrogen bonds with adjacent chains, which explains the change of the peak for Amide A to lower wavelengths as the result of an increasing number of hydrogen bonds [20]. The signal around 1530–1590 cm$^{-1}$ reveals the presence of Amide II [17], due to the bending for the N-H bond and the vibrational contributions because of the interaction between N and C [21]. Finally, the Amide III signal occurs at 1250–1260 cm$^{-1}$ [5], as a results of the combination of the bending of the NH group and vibrational stretching of CN [22].

Furthermore, the low frequency signal for amide V can be identified, which is not observed in collagen without dialysis, close to 650 cm$^{-1}$, usually identified in a frequency range from 575 to 775 cm$^{-1}$, attributed to the wave motion of the N-H bond and mainly to the movements of the CH$_2$ links [23].
There is a clear difference in the collagen without dialysis: besides an overall increase on the spectra, it presents two pronounced peaks at 1540 cm$^{-1}$ and 1410 cm$^{-1}$. This fact indicates that NaOH, necessary for crosslinking the fibers, reacts with the acetic acid, producing sodium acetate, whose representative peaks are 1560 cm$^{-1}$ and 1413 cm$^{-1}$ [24, 25], corresponding to the stress frequency of carbonyl group; which in turn is deposited into the self-assembled fibrils. Also, the band related to the CO bond close to 1090 cm$^{-1}$ is more intense in the collagen without dialysis due to the presence of COO-Na +, as well as the signal that is in 800 cm$^{-1}$ from the mineral phase and is only present in this spectrum.

Freeze-dried and dialyzed collagen show a small difference, barely noticeable on the changes of frequency, which are smaller for the dialyzed collagen in some absorption bands and can be caused by the conformational transitions in the structures [5]. These results show that the collagen composition was not affected by the self-assembly method used. They also demonstrate that the dialysis process after self-assembly was effective.

Calorimetry curves (DSC) for freeze-dried, dialyzed and undialyzed collagen were compared to examine the thermal properties and stability of the protein. Figure 3 shows a denaturalization temperature of 85 °C for undialyzed collagen, where the triple helix is opened [26]. Another transition temperature is observed around 60 °C, associated to the fusion of sodium acetate trihydrate (CH$_3$COONa) [27]. This signal is absent in the dialyzed collagen, which shows that the purification steps of dialysis eliminated the salt remains. The signal is also absent in the freeze-dried collagen, because it was not gelated and therefore never exposed to NaOH.

The denaturalization temperature is also reported in similar values: 85 °C for dialyzed collagen, and slightly lower for freeze-dried collagen, close to 75 °C [28].

There is a correlation between the thermal stability of collagen and the content of imino acid (proline and hydroxyproline) through hydrogen bonds [29] so that a higher content of imino acid of collagen is associated with a higher thermal denaturation [20]. However the content of amino acids is the same in lyophilized and self-assembly collagen. So the slight difference in the denaturation temperature between those collagens is that the self-assembled collagen has a micro fibrillar structure composed of several highly-arranged triple helices interacting by hydrogen bonds and forming those microfibers. In contrast the lyophilized collagen has a set of triple non-ordered helices. Hence the opening of the triple helix when it is interacting by hydrogen bridges and is highly aligned and ordered, needs more energy (higher temperature) to be the opening of the helix (denaturalization temperature).

In addition, endothermic category transitions indicate that higher enthalpies are required for collagen without dialysis and freeze-dried, in comparison with the dialyzed collagen, owing to the presence of salt and moisture respectively, substances that lead to an increment of energy to remove them. In the case of water, substantial levels of this content in the fibers network can decrease linearly the denaturation temperature [30].
The dialyzed collagen also absorbs moisture from the environment, but in a minor amount because of the form of the samples (dry films) compared with freeze-dried collagen [31].

It is also important to consider that heat capacity of collagen depends on its acidity and its concentration [32].

The presence of sodium acetate can also be seen in the SEM images, in collagen without dialyzing shown in Fig. 4 (a). Some crystal formations can be appreciated, they are absent in the dialyzed collagen shown in Fig. 4 (b). In the dialyzed collagen, it can be hardly observed light spots, traces of salt. The fibrillar structure can be seen in both types of collagen, with one higher porosity in the dialyzed collagen, an important feature for a biomaterial [6].

The morphology of the self-assembled collagen fibers can be seen in detail in Fig. 5, showing images of atomic force (AFM) microscopy.

The microfibrils of collagen form a pattern with a period (D-periodic) of 65 nm. This value can be find within the characteristic measures of 64 to 67 nm [3] and corresponds to the displacement of each molecule in the axial direction, with respect to the adjacent molecule [33]. It could be considered that this variation in the periodic spacing is intrinsic to the collagen type I self-assembly process, because of local changes in the mechanical stresses. Those changes are due to the variations in the intra-fibrillar interactions, including hydrophobic and electrostatic interactions, hydrogen bonds and crosslinks in hydroxylysine and hydroxyprolines [34]. Each fibril diameter is between 450 and 550 nm, which coincides with the reported value for the tendons fibrils that are up to 1 cm long and 500 nm in diameter [35].

The stress-strain curves of collagen type I in Fig. 6 show a characteristic non-linear elastic behavior, response that can be attributed to the straightening of the conformations of the triple helix [36] and the alignment of the N - and C - terminal of the crosslinking [37].

Undialyzed collagen exhibits an ultimate tensile strength of 9.06 MPa, much higher than the strength obtained for the dialyzed collagen, of 2.38 MPa. This is due to salt that offers greater rigidity to the collagen that has not been purified, through chemical interactions that can generate cross-linking points. This in turn makes the molecules more rigid, since the relaxation movements are disabled, increasing the collagen tensile strength [38].

In this study, the self-assembly process of collagen was studied titrating a solution of acetic acid - collagen with NaOH (comments from graphics). Acid –base titrations by ITC are commonly carried out for calibration [39] but in this case was intended to determine the pi due to the absorption or release of heat due to the collagen assembly process. The assembly depend on pH, temperature, and protein concentration. The temperature was maintained constant during the experiment and the protein concentration used was higher than the minimal critical concentration for assembly [40].

Figure 7a shows the neutralizing reaction without the collagen in the cell. Figure 7b shows the heat released due to the dilution of the collagen with acetic acid as a control experiment. The dilution didn´t
produce a high energy release of disassembly according to the assembly values obtained. Figure 7c and d shows the release or absorption of energy due to the increase in pH and ion concentration on the collagen and gelatin acetic acid solutions, respectively. There are observed differences in the energy release rate between the collagen and gelatin, suggesting a different assembly process. Two experiment were carried out to determine if the release or absorption of energy were mainly influenced by the agglomeration due to a salting in or salting out effect and not due to the molecular interactions involving proton release or absorption of the macromolecule leading assembly. The released energy values were small due to the addition of the NaCl or sodium acetate buffer to the collagen-acetic acid solution, suggesting that the energy release or absorption was driven for intermolecular interactions and not due to precipitation related to high concentration of ions. However, the effect in the equilibrium conditions due to their effect on the bulk water activity is still present for each ion pair. They may influence the electrostatic interactions between charged macromolecules participating in binding events, Debye–Hückel screening effects, or change of water activity [41].

Figure 8a shows the area under the curve in relation to moles for the base solution over the acid and salt and Fig. 8b shows the enthalpy changes due to pH increase. The release of energy maintains similar values until the pH reaches values close to the pl. The area under the curve in relation to moles for the base solution over the acid collagen solution and for the base solution over the collagen gelatin are shown in Fig. 8c and Fig. 8d respectively.

Some studies reported that the fibrillogenesis of collagen dilutions occur above pH 5, the pre-assembly of their triple helices in poorly organized structures can begin from lower pH values and high ionic strengths [42].

The steepest increase in the ΔH was observed at a pH value of 6.5. The negative ΔH shows an exothermic process with higher energy release values compared with the obtained for the injection of different buffers to the acetic acid solution, referring to changes for dilution and ionic strength. F. Jiang et al. showed the pH influence on the collagen self-assembly, collagen molecules were adsorbed under hydrodynamic flow onto mica at pH values ranging from 2.5 to 10.5 and keeping constant the electrolyte concentration and next imaged by AFM. Between pH 2.5 and 3.5 they observed elongated globules no pronounced fibrillar structures were observed but between 5.5 and 9.5 the fibrillar structures were presented in the mica surface. At the isoelectric point the collagen was self-assemble with an associated enthalpy value of 33,89 mJ for 0,01035 moles of collagen and the structure was maintained at higher pH [43]. This information is of vital importance in tissue engineering and especially in organ bio-printing 3D, which has become a technique quite used today, to set the parameters of the operation according to the endothermic transitions.

To determine if the enthalpy obtained is due to fiber self-assembly the collagen was denatured and the same process of neutralization was done. The DSC thermograms after denaturalization are shown in Fig. 3. In both cases, care was taken to use the same protein concentration and the same acid and base solution and low viscosity solutions were used for avoiding easy precipitation of the gelatin. A steeper
enthalpy change is obtained at the same pH. However, for gelatin an endothermic peak was observed at the pI, showing different assembly process and gelification between the gelatin and the collagen.

The nucleation growth mechanism of collagen fibril assembly has been reported to be driven by a polymerization reaction, starting from the monomer and because of the possible presence of covalently crosslinked oligomers [40], with the intervention of both, hydrophobic and ionic inter-collagen interactions [44]. Protein molecules are random coiled (denatured) in non-aqueous medium. Polar residues seek to form hydrogen bonds and therefore create nonpermanent α-helices and β-sheets [45].

To conform the gelatin, it is necessary to break up the secondary and higher structures of the parent protein collagen, with varying degrees of hydrolysis of the polypeptide backbone [46], from a random crosslinking of primary chains, locally twisted together, so the aggregation process could be driven by the hydrophobic effect [47].

Conclusions

With the results obtained from this study it was demonstrated that Type I collagen can be obtained from animal sources with stable properties and very similar characteristics to type I collagen present in the human body.

Using FT-IR and CD studies, it was corroborated that collagen is not suffering significant compositional changes through the self-assembly process, confirming the presence of pPII in rat tail tendon collagen and a correct structure related to the triple helical structure and 4R-hyp. However, features may vary, depending on the gel washing process. If the gel is not dialyzed, remains of sodium acetate can be appreciated in the collagen fibers, in the SEM images.

The denaturation temperature of the collagen also varies slightly. A denaturation temperature of 85 °C was obtained for the dialyzed collagen, and regarding to the structure, self-assembly collagen fibrils present a periodic gap of 65 nm, as expected from type I collagen morphology. In addition, the salt residue can affect the tensile strength, generating a significant increase in the result.

Using the stoichiometric and calorimetric analysis of the collagen self-assembly, it is determined that the self-assembly process starts from a 4.4 pH and the self-assembly heat is 7159.61 mJ/mol. The study of these properties of self-assembly is important, not only to develop products based on collagen scaffolds in tissue engineering applications, but also for modifying their physical-chemical properties.

The most common experiments related to the formation of oligomers are related to dilution experiment. However, in this study the aim was to demonstrate the self-assembly and aggregation of the collagen molecule due to the pH change.

Abbreviations
Declarations

- Ethical approval and consent: This article does not contain any studies with human participants or animals performed by any of the authors. For this type of study, formal consent is not required.
- Consent for publication: Not applicable.
- Availability of data and materials: All data generated and analyzed during the current study are available from the corresponding author on reasonable request.
- Competing interests: All authors of this article declare that have no conflict of interest.
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- Authors' contributions: All authors designed and performed the experiment, analyzed the data, interpreted the study results and drafted the manuscript. JGM, JCS, RGP, NMU designed and supervised the experimental works, interpreted the study results and reviewed and finalized the manuscript. JGM, JCS, RGP, YRCU, JRVB contributed to study design and improved the manuscript. All authors read and approved the final manuscript.
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Figures
Figure 1

Circular dichroism (CD) spectra of collagen extracted from rat tail tendon.
Figure 2

Fourier transformed infrared spectroscopy (FTIR) spectra of extracted collagen in different conditions: freeze-dried and self-assembled: dialyzed and undialyzed.
Figure 3

Differential scanning calorimetry (DSC) thermogram of rat tail tendon collagen type I in different conditions: freeze-dried and self-assembled: dialyzed and undialyzed.
Figure 4

Scanning electron microscopy (SEM) images of (a) non-dialysate collagen and (b) dialyzed collagen; 100 µm scale, 1.0k.

Figure 5

Atomic force microscopy (AFM) self-assembled collagen images in the mode of (a) Height 10 µm, (b) Amplitude 10 µm, (c) Height 2.5 µm, (d) Phase 2.5 µm.
Figure 6

Stress-strain curve of self-assembled collagen type I: dialyzed and undialyzed.
Figure 7

Heat aggregation of extracted collagen by isotherm titration calorimetry (Nano ITC) sensed from the injection of: a) the base solution over the acid, b) the acid solution over collagen, c) the base solution over the acid collagen solution, d) the base solution over the collagen gelatin.
Figure 8

Gelation thermodynamics of extracted collagen by isotherm titration calorimetry (Nano ITC) sensed from the injection of: a) the base solution over the acid and salt, b) the collagen solution over acid solution, c) the base solution over the acid collagen solution, d) the base solution over the collagen gelatin.

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