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Method of addition of acetonitrile influences the structure and stability of collagen

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ABSTRACT

Collagen has been extensively used as a biomaterial in many biomedical applications. Recently, collagen based biomaterials were prepared using organic solvents. In this context, the method of addition of organic solvent described in the present study will be an important contribution in the preparation of collagen-based biomaterials. The effect of acetonitrile on collagen structure and stability was investigated using biophysical methods. Collagen undergoes solvent-induced denaturation with increasing concentration of acetonitrile. It was observed that addition of acetonitrile (50–90%) to a collagen solution in a single shot (method 1) led to precipitation. Contrary, collagen remained in the solution when acetonitrile content was increased to 90% in a collagen solution that had been formerly equilibrated with 20% acetonitrile (method 2). Interestingly, triple helical structure was retained when precipitated collagen, obtained from method 1, was re-dissolved in acetic acid solution. The re-dissolved collagen exhibits comparable melting temperature as that of native collagen. Re-dissolved collagen also showed fibril formation, but with decreased rate. The soluble collagen in 90% acetonitrile, prepared by method 2, is found to be unordered. The above results thus suggest that the method of addition of acetonitrile plays an important role in the folding and unfolding of collagen.

1. Introduction

Collagens are the most abundant proteins found in extracellular matrices of vertebrate animals. In animal hides and skins, the dominant collagen is type I and it is also the major structural component of tendon, bone and connective tissue [1]. Collagen exists in the form of fibrils and they provide the main mechanical support and structural organization of connective tissues. Because collagens provide natural structure, biodegradability, and biocompatibility, they have extensive applications as a biomaterial in tissue engineering, wound healing, as drug carriers, and cosmetics. For example, fibril-forming collagens provide a scaffold for cell attachment and migration, as well as providing specific mechanical properties [1]. Because of the presence of collagen, the animal-derived tissues are used for the replacement of human tissues that results in the improvement of the wound-healing process. Furthermore, it has been used as the main component in the design of biomaterials such as artificial dermis, wound dressings, tissue engineering devices, tendon substitutes, and injectable material in plastic surgery [2–6]. Collagen from animals, particularly from bovine species, is more advantageous due to the possibility of extraction of a large quantity of pure type I collagen. It is noteworthy that there are significant relationships between collagen and many diseases such as rheumatoid arthritis [7] and systemic sclerosis [8]. The structure and stability of collagen are thus an important factor as they are widely used as biomaterials.

Type I collagen consists of two α1(I) polypeptide chains and one α2(I) chain [9], and each chain contains about 1040 amino acids. Each chain is coiled into a left-handed poly-proline II (PPII) helical structure having a repetitive triplet sequence, Gly-Xxx-Yyy where Xxx and Yyy can be any of the amino acids, but are frequently occupied by imino acids viz., proline and hydroxyproline. These left-handed helices coiled together to form a unique right-handed triple helical structure [10,11] of about 3000 Å length and 15 Å diameter [12]. However, collagen also contains non-helical regions with 15–25 amino acids. The self-association of triple helices leads to fibrils with diameters of about 30–300 nm. It is believed that the specific functions of collagen are related to the hierarchical structure from triple helix to fibre. The structure and stability of this triple helix has been the subject of many studies. The major force which stabilizes the triple helical structure of collagen in solution is considered to arise from hydrogen bonds, which include both

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inter-chain [13] and water-mediated hydrogen bonds [11,14,15] between the polypeptides. In addition, the restriction of backbone rotation of the imino acid residues (proline and hydroxy proline) and hydrophobic interactions between the nonpolar residues on the surface of the collagen molecules contribute significantly to the stability of triple helix in solution. The forces which are responsible for collagen structure stability have been obtained from many studies, which include thermal denaturation of collagen in the presence of various small molecules and co-solvents in water [16–19], model collagen peptides [20–23] and modification of hydroxy proline residues in model collagen peptides [24–26], amino acid substitutions in triplet sequence [27], etc. It is noteworthy that co-solvents have been employed in the preparation of collagen-based biomaterials, namely collagen–glycosaminoglycan scaffold and collagen nano-fibres, which are used for tissue engineering applications [28–30]. The collagen-based biomaterials were prepared by electro-spinning method using organic solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) or 2,2,2-trifluoroethnaol (TFE) [28,31]. Recently, a detailed protocol for reproducible electrospun nano-fibre fabrication from collagen has been reported [32]. It is noteworthy that collagen underwent denaturation in both HFIP and TFE solvents [19,33]. Since organic solvent has been used for the preparation of collagen-based biomaterials, it is necessary to understand the behaviour of collagen (solubility and stability) in organic solvents. The current findings thus could be an important in the preparation of collagen-based biomaterial using organic solvents. Apart from biomedical applications, understanding of collagen–solvent interaction is extremely important for the processing industries for making leather, gelatine and other cosmetic applications.

Although several organic co-solvents were studied to understand the stability of collagen, all these studies were conducted with the maximum concentration of 50% (v/v) and most of them were alcoholic solvents such as mono and polyhydric alcohols [17,34–37]. In this report, we studied the effect of a non-alcoholic solvent such as acetonitrile (with a concentration range of 0–90% (v/v)) on collagen to understand the role of non-alcoholic solvents and also the effect of higher concentration of organic solvent on the structure and stability of triple helical structure. This knowledge is also essential for the development of new processing methodologies in handling collagen for various industrial applications.

2. Materials and methods

2.1. Materials

Acetonitrile was purchased from S.D. Fine-Chem Ltd. (India). Collagen was isolated and purified from tails of six-month-old male albino rats as reported previously [38,39]. The concentration of collagen was determined from the hydroxyproline content according to the method of Woessner [40].

2.2. Sample preparations

A stock solution of collagen was prepared in 50 mM acetic acid. A fixed volume of collagen stock solution was diluted with an appropriate volume of acetonitrile and 50 mM acetic acid to get the desired concentration of acetonitrile. Since collagen solubility in the presence of acetonitrile depends on the method of addition of solvents (vide infra), the solvents were added in the following order: collagen stock, 50 mM acetic acid, and acetonitrile. For acetonitrile concentration up to 30% (v/v), the required volume of acetonitrile was added in a single shot to a solution containing collagen stock and required volume of 50 mM acetic acid. At 90% acetonitrile, two methods were followed. In method 1, 90% of acetonitrile was added in a single shot to a fixed volume (10%) of collagen stock solution (see Scheme 1). In method 2, acetonitrile concentration was increased to 90% in a collagen solution, which had been previously equilibrated with 20% acetonitrile (see Scheme 1). The collagen precipitate obtained from method 1 was re-dissolved in 50 mM acetic acid solution at room temperature.

2.3. CD measurements

Far UV CD spectra of collagen in the absence and presence of different concentrations of acetonitrile in 50 mM acetic acid solution and the thermal melting of collagen in the presence of acetonitrile were measured using Jasco J715 spectropolarimeter. A rectangular quartz cell with a path length of 0.1 cm was used. The instrument was calibrated using ammonium-d_{10}-camphor sulfonate as described by the instrument manufacturer. Before CD measurements, the solutions were incubated (without stirring and shaking) at 25 °C for ~10 min. All spectra were recorded from 300 to 190 nm with standard sensitivity. The parameters such as wavelength scanning speed, band width, response, and data pitch were set at 100 nm/min, 1 nm, 1 s, and 0.5 nm, respectively. All CD spectra of collagen were solvent subtracted. Each CD spectrum represents the average of three individual scans. For thermal melting studies, collagen solution, with and without acetonitrile, was taken in 0.1 cm rectangular quartz cell. The temperature was increased from 20 to 60 °C at a rate of 1 °C/min. The midpoint of the transition cure was taken as melting temperature of collagen. The temperature of the cell holder was controlled using a Peltier temperature control accessory supplied by Jasco Inc. The other experimental parameters were set as described above.

2.4. FTIR measurements

All FTIR spectra were recorded using FTIR spectrometer (ABB MB3000). About 200 μL of the collagen solution containing different volume of acetonitrile, which was incubated for ~10 min, was cast onto a ZnSe plate that was placed at 25 °C. The sample was incubated until a dry thin film formed on the surface of the ZnSe window. For control, collagen film was derived from 50 mM acetic acid. In the case of the collagen precipitate obtained by method 1, the precipitate was placed on ZnSe plate and dried. All FTIR spectra were baseline corrected. Each spectrum was collected for 20 scans at a resolution of 8 cm^{-1}.

2.5. Fluorescence measurements

Fluorescence spectra were obtained on a Perkin-Elmer LS-45 spectrometer at room temperature, using a quartz cell with path length of 1 cm. Before measurements, the sample solutions were incubated for ~10 min at room temperature. For denatured collagen fluorescence, the native collagen solution was heated to 60 °C and the denaturation of collagen was confirmed by CD. The excitation wavelength was fixed at 270 nm. The emission spectra were recorded between 290 and 400 nm with the scan rate of 100 nm/min. The bandwidth for excitation and emission light was 10 nm.

2.6. Fibril formation studies

The self-assembly of collagen (fibril formation) was monitored at 313 nm using Perkin-Elmer UV-vis spectrometer (Lambda EZ 201) at 27 °C. At the time of measurements, 100 μL of native collagen solution (50 mM acetic acid), 800 μL of water, and 100 μL of 10× phosphate buffer saline (PBS) (pH 7.2) was added sequentially. After addition of water, the resultant collagen solution was