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# 2,2,2-Trifluoroethanol disrupts the triple helical structure and self-association of type I collagen

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ABSTRACT

Collagen, a fibrous structural protein, is a major component of skin, tendon, bone, and other connective tissues. Collagen is one of the dominant biomaterials used for tissue engineering and drug delivery applications. 2,2,2-Trifluoroethanol (TFE) has been used as a co-solvent in the preparation of collagen based biomaterials, which are used for tissue engineering applications. However, the basic knowledge about the structural behavior of collagen in TFE is necessary for an adequate application of collagen as a carrier system. In this work, the effect of TFE on the structure and self-association of collagen has been studied in detail using different spectroscopic methods such as circular dichroism (CD), Fourier transform infrared (FTIR), and UV–Vis absorption. The results obtained from CD and FTIR suggest that collagen transform its structure from triple helix to predominantly unordered conformation with increasing concentration of TFE. Thermal melting studies reveal that the stability of collagen triple helix decreases even at low concentration of TFE. Turbidity measurements indicate that TFE, at higher concentrations, inhibits the collagen fibril formation which arises due to the self-association of collagen molecules. TFE has conventionally been known to promote the ordered structures in proteins and peptides. Destabilization of collagen triple helix by TFE is first of its kind information on the effect of TFE to disrupt the native conformation of proteins.



## 1. Introduction

Collagen, the most abundant fibrous protein accounts for the structural integrity of vertebrates and many other multicellular organisms. It has unique structure in which three polypeptide strands, each possessing left-handed polyproline II-type (PPII-type) helix, twisted together into a right-handed coiled coil structure, named as triple helix [1,2]. The presence of glycine at every third residue in a Xaa-Yaa-Gly (where Xaa and Yaa can be any amino acid) repeating motif makes it possible for the existence of a tightly packed triple helical structure in collagen. It has been well established that the major forces stabilizing the collagen triple helices are hydrogen bonding. The presence of extensive inter-strand hydrogen bonding network between N—H Gly of one strand and O=C Xaa of neighboring strand plays a major role in the stabilization of triple helix [3]. The orientation of side chains of the Xaa and Yaa residues are also important as they participate in intra and intermolecular side chain interactions [4]. Further, hydroxyproline (Hyp) residues plays a significant role, as it is suggested to form water mediated bridges with free carbonyl groups in the triple helix [5]. The temperature-favored assembly of collagen was also driven by water-mediated hydrogen bonding between polar residues [6]. However, recently, Shoulders et al. demonstrated that Hyp residues stabilize the collagen triple helix via a stereo electronic effect that organizes appropriate backbone torsion angles for triple-helix formation [7].

Collagen-derived products as biomaterials have a tremendous impact in biomedical applications because the natural collagen triple helical structure act as a biological support for cells and scaffold for tissue repair and regeneration. One of the biomaterials namely collagen-glycosaminoglycan scaffold, which is used for tissue engineering applications [8,9], has been prepared from aqueous 2,2,2-trifluoroethanol (TFE) mixture. TFE has also been used for the preparation of collagen nano-fibers by electro-spinning, the most fruitful method to manufacture in vitro fibrous scaffolds for tissue engineering application [10]. Although TFE has been successfully applied for the preparation of biomaterials, the basic knowledge about the structural behavior of collagen in TFE is necessary for an adequate application of collagen as a carrier system. More than three decades, TFE has been known to be a structure

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making solvent in proteins and peptides [11]. It has been shown that TFE induces and stabilize predominantly α-helical conformation in peptide sequence, which has intrinsic helical propensity [12]. Structure with turns, β-hairpins, β-strands and $3_{10}$-helical conformations are also possible in TFE [12–14]. Further, it can stimulate the conformational transition between different secondary structures such as β-sheet → α-helix [15], random coil → α-helix [16], and random coil → β-sheet [17] in proteins and peptides. Random coil to type II β-turn transition has also been reported for an elastin peptide in TFE [18]. It is noteworthy that TFE mimics the local decrease of dielectric constant in the vicinity of a membrane surface [19]. Several mechanisms have been proposed for the interaction of TFE with protein. First, the preferential binding of TFE to the backbone carbonyl oxygen of the amino acid can lead to enhance intra-molecular hydrogen bonding due to the minimization of solvent exposure of the peptide bond [20]. Second, TFE preferentially binds with hydrophobic sites on the proteins [21] and peptides [22] thereby weakening hydrophobic–hydrophobic interactions. Third, the structure of water is greatly affected by TFE [23,24]. Recently, a desolvation model has been proposed for the TFE induced aggregation of green fluorescent protein [25].

In this work, the effect of TFE on the structure of type I collagen has been examined using spectroscopic techniques such as circular dichroism (CD), Fourier transform infrared (FTIR), and UV–Vis absorption. The results prove that collagen undergoes triple helical to unordered conformation with increasing concentration of TFE. The triple helical stability of collagen has been significantly affected even at low concentration of TFE.

## 2. Materials and methods

TFE (spectroscopic grade) was purchased from Spectrochem Pvt. Ltd. (India).

### 2.1. Extraction and purification of type I collagen

Collagen fibers were teased out from tails of six-month old male albino rats (Wistar strain) and thoroughly washed with 0.9% saline water at 4 °C, to remove the adhering muscles and other soluble proteins. Acid soluble type I collagen from rat tail tendon (RTT) was isolated according to the method as described earlier [26,27]. The procedure includes collagen extraction using acetic acid and salting out with NaCl. The collagen purity is confirmed by SDS–polyacrlyamide gel electrophoresis. The collagen concentration in the solution was estimated from the hydroxyproline content according to the method of Woessner [28].

### 2.2. Circular dichroism measurements

Far UV CD spectra of collagen, with and without TFE, at 25 °C were measured using J715 CD spectropolarimeter, using a 0.1 cm rectangular quartz cell. The CD instrument was calibrated using ammonium-$d_{10}$-camphor sulfonate as described by the instrument manufacturer. A stock solution of collagen was prepared in 50 mM acetic acid solution. For TFE titrations, appropriate volume of TFE and 50 mM acetic acid solution was added to the constant volume of collagen solution. Before CD measurements, the solutions were incubated (without stirring and shaking) at 25 °C for ~10 min. The CD spectra were recorded from 300 to 190 nm with wavelength scanning rate of 100 nm/min. The band width, data pitch, and response time were set at 1 nm, 0.5 nm, and 1 s, respectively. The solvent spectra were used as baselines, which were subtracted from the collagen CD spectra. The spectra reported are the average of three scans. Thermal melting of collagen in the absence and presence (10 and 15%, v/v) of TFE were measured concurrently. The temperature was increased from 5 to 60 °C at a rate of 1 °C/min. The temperature of the cell holder was controlled with a peltier (six cell holder) temperature control accessory provided by Jasco, Inc. Other parameters were set as described above.

### 2.3. FTIR measurements

All IR spectra were recorded by a normal transmission mode using FTIR spectrometer (ABB MB3000). About 100 μl of the collagen solution, prepared in 50 mM acetic acid, was cast onto a ZnSe window that was placed at 25 °C. The sample was incubated until a dry thin film formed on the surface of the ZnSe window. Similar procedure was adopted for the collagen solution containing different amount of TFE. All FTIR spectra were collected for 20 scans at a resolution of 8 $cm^{-1}$. The FTIR spectra were baseline corrected. Fourier self-deconvolution of the amide I spectral region was done using Grams/AI (v 7.00) from Thermo Galactic. The spectra were deconvoluted using a gamma factor 9 and half width half height of 18 $cm^{-1}$.

### 2.4. Fibril formation studies

The process of self-association of collagen was observed at 313 nm using CARY 100 UV Visible spectrophotometer at 37 °C using CARY dual cell peltier accessory. At the time of measurements, the stock solution of collagen was diluted to a final concentration of 0.4 mg/ml by the addition of appropriate volume of water, TFE and 10× PBS (pH 7.0) in the quartz cuvette with the path length of 1 cm. Briefly, the collagen and water were taken in a cuvette, and the resulting solution was incubated with appropriate volume of TFE for 5 min in closed conditions [29]. The PBS was added just before the kinetic measurements. The final pH of the solution was pH 6.0. The collagen in the absence of TFE was used as a control. The reference solvents for every experiment were prepared with an appropriate amount of water, TFE and buffer without collagen to normalize the background.

## 3. Results and discussion

### 3.1. Effect of TFE on the structure of collagen

CD provides a rapid method to assess the conformation of proteins and peptides in solution. We have used CD to monitor the secondary structural changes of type I collagen induced by TFE. The solvent concentration dependence of the CD spectra of collagen in TFE (0–90%, v/v) is shown in Fig. 1. As expected, the CD spectrum of collagen, in the absence of TFE, shows a positive band at 222 nm followed by a strong negative band at 198 nm, which are diagnostic of left-handed PPII-type helix structure. This result demonstrates that collagen exits in native conformation with triple helical structure. At 10% TFE (v/v), the CD spectrum did not change significantly, indicating that PPII-type conformation of collagen is not affected by 10% TFE solution (Fig. 1). Further increase in the concentration of TFE to 15% (v/v), the CD spectrum exhibits a decrease in the positive ellipticity at 222 nm and negative ellipticity at 198 nm, suggesting that the formation of triple helix decrease with increasing TFE concentration. When the TFE is increased to 20% (v/v), the positive ellipticity of collagen at 222 nm disappeared and the negative ellipticity at 198 nm reduced drastically. The appearance of a single negative band at 198 nm is normally associated with random coil structure. However, in the literature, the terminology of random coil and unordered structures is used as a misnomer. Nevertheless, recent reports clearly demonstrate that random coil structure is to be considered to have local dihedral angles as in PPII structure but without long range order or to be a disordered structure with local stretches of left handed helices [30–32]. The random coil structure is now referred as “PPII-type” structure. Consequently, the PPII and