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ARTICLE *in* INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES · JULY 2012

Impact Factor: 2.86 · DOI: 10.1016/j.ijbiomac.2012.06.039 · Source: PubMed

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Dielectric behaviour and conformational stability of collagen on interaction with DNA

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ARTICLE INFO

Article history:
Received 27 April 2012
Received in revised form 25 June 2012
Accepted 26 June 2012
Available online 3 July 2012

Keywords:
Collagen
DNA
Circular dichroism (CD)
Impedance
Viscosity

ABSTRACT

Collagen–DNA interaction studies will aid in improving the stability of DNA against nucleases. In the present study, the effect of DNA on different physico-chemical properties of collagen like viscosity, conformation and dielectric behaviour has been studied. Increase of DNA concentration leads to the increment of viscosity of collagen at the pH 4 and 5, but the trend is reversed at the pH of 6 and 7 due to the formation of collagen fibrils. The temperature dependent CD spectroscopic studies for collagen–DNA conjugate showed that thermal stability of collagen is modulated with increasing molar concentration of DNA. It also shows that DNA interactions with collagen did not result in change in the triple helical structure of collagen. Impedance measurements show that the strength of ion pairs for different molar concentrations of collagen–DNA conjugates has changed. Nyquist plot for collagen–DNA conjugate posses higher Y'' at DNA concentration of 1:25 and 1:50 whereas at 1:1 and 1:10 lower Y'' than the native collagen have been observed. An understanding of this nature of the collagen–DNA interactions is helpful for gene delivery applications.

1. Introduction

Collagen is one of the most commonly used biomaterials with broad applications in drug delivery, tissue engineering, scaffolds and wound dressing. Understanding the mechanism of stabilization of collagen against biodegradation and heat is necessary because it has far reaching implications in both industrial and biological applications of collagen. Collagen is a protein, which has coiled coil structure consisting of three polypeptide chains, two α_1 (1) and one α_2 (1) chain, intertwined to form a right handed triple helix having a molecular weight of 300 kDa, a length of 300 nm and a diameter of 1.5 nm [1]. The biodegradation rate, mechanical and thermal stability of native collagen are not sufficient for many *in vivo* and *in vitro* applications. Crosslinking of collagen is a way to improve the mechanical, thermal stability and to slow down the biodegradation rate for use in drug and gene delivery systems [2–5].

Crosslinking of collagen triple helix and double helix of nucleic acids is crucial for gene therapy. The problem in gene therapy and drug and gene delivery systems is that, after the gene vector containing DNA is applied into the living body, it gets deactivated due to immunological reaction or enzymatic attack. There is an uncontrollable expression of gene that is introduced into cell due to the attack. This is the same problem that is encountered in delivery of bioactive proteins that are unstable in the living body. If a system in which a gene vector is embedded in inactive biomaterial is administered into the living body, then the gene vector and bioactive protein can be protected from immunological and enzymatic attack. Thus, the release of the gene vector from the biomaterial and the gene expression period can be controlled [6–8]. Hence, it was found that DNA double helix and collagen triple helix will create a supramolecular self-assembly system in aqueous solution that will cause the rise in stability of DNA against the nucleases as well as the collagen developing the autoimmune reaction [9].

The influences of various factors on the stability of collagen have been widely studied [10–13]. It is recognized that both intermolecular and intramolecular interactions contribute to the specificity of protein–DNA recognition and their relative contributions vary depending upon the protein–DNA conjugate [14]. Recently, it has been shown that collagen can well preserve DNA, which means that DNA can be released from the nucleus and can be placed in the matrix between the cells in the connective tissue. The formation of collagen–DNA conjugate determines the stability of DNA against nucleases [7,8]. Recently, ATR-FTIR, AFM and NMR experiments were performed to study the mechanism of unusual collagen aggregation in the presence of DNA. It has been shown that collagen is protected from denaturation during the formation of collagen–DNA conjugate and could theoretically predict the possibilities of formation of hydrogen bonds between the peptide and DNA groups [6].

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http://dx.doi.org/10.1016/j.ijbiomac.2012.06.039

Although studies on collagen–DNA interaction have been carried out, the influence of this interaction on the dielectric and conformational behaviour of collagen is yet to be studied. Circular dichroism spectroscopy (CD) is a valuable tool to study the conformational stability of proteins [15]. Dielectric spectroscopy is a technique that is used to study the effects of hydration and electric field frequencies on the dielectric properties of constituent phases of unmodified collagen [16–19]. The main objective of this work is to investigate the influence of calf thymus DNA (CT–DNA) on the dielectric, thermal and conformational stability of collagen.

2. Materials and methods

2.1. Isolation and characterization of collagen type I

Tails were excised from 6-month-old albino rats (Wistar strain) and frozen at 253 K. On removal of freezer, tails were thawed and tendons were teased out. This acid soluble RTT collagen type I was isolated according to the method described by Chandrakasan et al. [20]. Acetic acid extraction and salting out with NaCl is included in the procedure. The stock solution of collagen was maintained at pH 4. The purity of collagen was confirmed by SDS-polyacrylamide gel electrophoresis. The concentration of extracted collagen was estimated from the hydroxyproline content using Woessner method [21].

2.2. Preparation and characterization of CT–DNA

A solution of CT–DNA in the Tris buffer of pH 7.3 gave a ratio of UV absorbance at 260 nm and 280 nm of about 1.8–1.9, indicating that the DNA was sufficiently free from protein [22]. The DNA concentration per nucleotide was determined by absorption spectroscopy using molar absorption coefficient 6600 M^{-1} cm^{-1} at 260 nm [23].

2.3. Sample preparation

Working concentration of collagen was taken as 0.202 μM at the pH 4. The aqueous solution of collagen–DNA conjugates was prepared at the different molar concentration ratios (1:1, 1:10, 1:25, and 1:50) and at different pH (4, 5, 6 and 7). The pH was maintained using 0.2 mol/L citric acid–disodium hydrogen orthophosphate buffer. The concentration of collagen was kept constant and DNA concentration was varied.

2.4. Viscosity measurements

Viscosity measurement was carried out to find the effect of DNA on the rheological property of native protein. Viscosity was measured using an Ostwald type viscometer. The viscometer was thermo stated at 298 ± 1 K. The flow times of aqueous solutions of collagen–DNA conjugate at different concentration ratios and at different pH were measured after a thermal equilibrium time of each sample for 30 min. The viscosity measurement was based on the flow rate of collagen solution through the capillary of an Ostwald viscometer. The flow time of protein with and without DNA was measured with a digital stopwatch at least three times and the average was taken. The specific viscosity (Pa-s) which expresses the incremental viscosity of the sample due to the presence of another solutes in the solution was calculated from the relation,

$$\eta_{sp} = \frac{t - t_o}{t_o} \quad (1)$$

where t and t_o are the flow times of samples and buffer, respectively. Relative viscosity (η/η_o) was calculated by Eq. (2)

$$\eta_{\text{relative}} = \frac{\eta}{\eta_o} \quad (2)$$

where η and η_o are the viscosity of collagen in the presence and absence of DNA. Normalizing η_{sp} to concentration c (μmol/L), *i.e.*, η_{sp}/c expresses the capacity of DNA to alter the protein solution viscosity; *i.e.*, the alteration of viscosity per unit concentration of DNA. η_{sp}/c is plotted as a function of the [DNA]/[collagen]. All experiments were carried out in triplicate.

2.5. Temperature dependent CD-spectroscopic studies

Influence of DNA on the temperature induced conformational changes of protein was studied at the pH of 4 using Jasco-815 spectropolarimeter at a temperature range of 303–313 K an interval of 1 K in the far UV region (190–280 nm) under N_2 atmosphere. The quartz cell with a light path 1 mm with 0.2 nm intervals was used for measuring the molar ellipticity of the protein. The samples were equilibrated for 30 min and the computer-averaged trace of 3 scans was employed in all calculations. Signal due to solvent was subtracted. The data were normally plotted as molar ellipticity (unit: degrees cm^2 $dmol^{-1}$) *versus* wavelength in nm. The molar ellipticity of a given solute at wavelength λ($[\theta]_{\text{molar}\,\lambda}$) is given by

$$[\theta]_{\text{molar},\lambda} = \frac{100 \times \theta_{-\lambda}}{m \times d} \quad (3)$$

where $\theta_{-\lambda}$ is the observed ellipticity (degrees) at wavelength λ and d is the pathlength (cm), m is the molar concentration of the solute. All experiments were carried out in triplicate and average values are reported.

2.6. Dielectric measurements

AC impedance analysis was carried out to determine the effect of DNA on the resultant dipole of the protein molecule responding to an alternating electric field by means of CH Instrumental (USA) electrochemical analyser CH-model 660B with a classical three-electrode system, where the glassy carbon electrode was used as a working electrode, a platinum electrode as a counter electrode and a saturated calomel electrode as the reference electrode. The collagen–DNA conjugates at the pH of 4 were used. The operating conditions of dielectric were Init E (V) = 90 mV, high frequency (f) = 10^5 Hz, low frequency (f) = 0.01 Hz, amplitude (V) = 0.005, quiet time = 2/s, cycles (0.1–1 Hz) = 1, cycles (0.01–0.1 Hz) = 1, cycles (0.001–0.01 Hz) = 1. All measurements were done in triplicates and average values are reported.

Impedance Z (unit: Ω) is measured as the voltage drop when a low-level sinusoidal current is injected into the biological sample. Dielectric data can be represented in terms of admittance. The admittance Y^* (unit: Ω^{-1}) is a conjugate quantity and may be written as

$$Y^* = Y' + jY'' \quad (4)$$

where Y' is the real component describing the energy stored and Y'' is the imaginary component depicting the energy dissipated by the system. All measurements were repeated for 3 times for reproducibility.

3. Results

3.1. Viscosity analysis of the collagen–DNA conjugate

The effect of CT–DNA on the viscosity of collagen was studied. The relative viscosity was measured for collagen–DNA conjugate

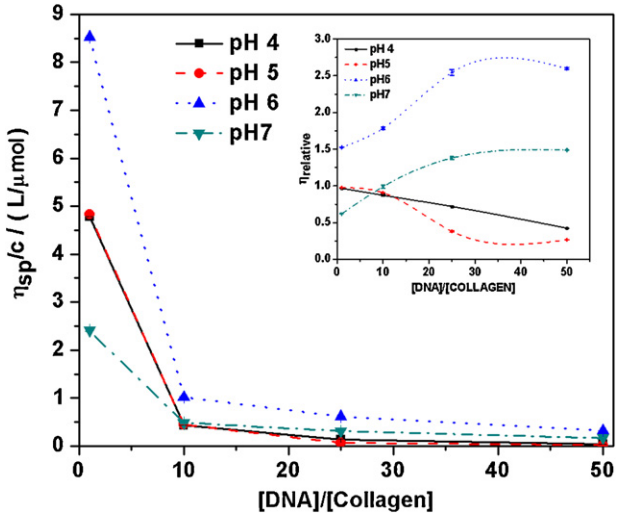

Fig. 1. A plot of normalized viscosity (η_{sp}/c) and relative viscosity η_{relative} (η_{sp}/η_o) against $1/R$ (c is the concentration of CT–DNA in μmol/L, R = [collagen]/[CT–DNA]). *Conditions*: Temperature = 25 ± 1 °C.

at varying concentrations and pH. The plot of normalized viscosity (η_{sp}/c) as well as relative viscosity η_{relative} (η_{sp}/η_o) against $1/R$ (R = [collagen]/[CT–DNA]) has been shown in Fig. 1. At the pH of 4 and 5, there is a decrease in the relative viscosity with the increase in the CT–DNA concentrations and for the pH of 6 and 7, it was found that there was an increase in the relative viscosity with the increase in the CT–DNA concentrations. The decrease of normalized viscosity (η_{sp}/c) with increase in DNA concentration for the collagen–DNA conjugate is a result of a decrease in effective protein size due to reduction of charged group repulsion by shielding of the cationic charges of protein as a result of the proximity of the anionic charges of DNA.

3.2. Temperature dependent CD-spectroscopic studies

In the far UV region, there is a negative peak obtained at 190 nm and a positive at 220 nm with a cross over at 210 nm for the triple helical conformation of collagen [24]. The molar ellipticity values of native collagen and collagen–DNA conjugate at different molar concentrations and at varying temperature range are shown in Fig. 2. The parameter Rpn denotes the ratio of positive peak intensity over negative peak intensity. It is a characteristic ratio for the triple helical conformation of collagen and collagen like peptide [25]. The Rpn values for native collagen and collagen–DNA conjugate at varying temperatures are given in Table 1. The Rpn ratio for different concentrations does not have a large variation even at the varying temperatures.

3.3. Dielectric measurements

The changes in the dielectrical behaviour of collagen on interaction with DNA were studied and the Nyquist plots and Bode plots are shown in Fig. 3a and b. At higher DNA concentration, *i.e.*, 1:25 and 1:50, Nyquist plot for collagen–DNA conjugate posses higher Y'' than the native collagen and at [collagen]/[DNA] concentration of 1:1 and 1:10, lower Y'' than native collagen is observed.

4. Discussion

The stability of 3D structure of double helical DNA is influenced by H-bonds between complementary base pairs (A = T, G≡C), stacking interactions along the helix axis and interactions with surrounding water layers. The assembly of different forms (A-, B-, Z-)

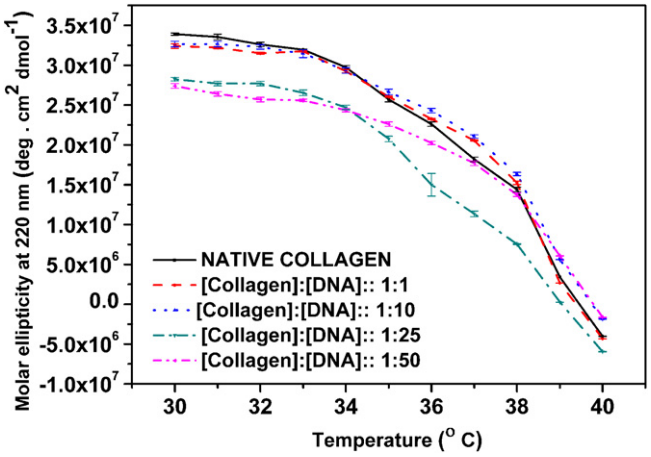

Fig. 2. Plots of temperature (°C) *versus* molar ellipticity value for collagen–DNA conjugates at 220 nm.

of ds-DNA and their conformational dynamics are also affected by the bound water in the multilayer hydration shell of the double helix [26]. Most principal conformation of DNA in aqueous solution is B form. It is known that Z-DNA has 40% higher viscosity than B-DNA [27]. The complete conformational changes of CT–DNA

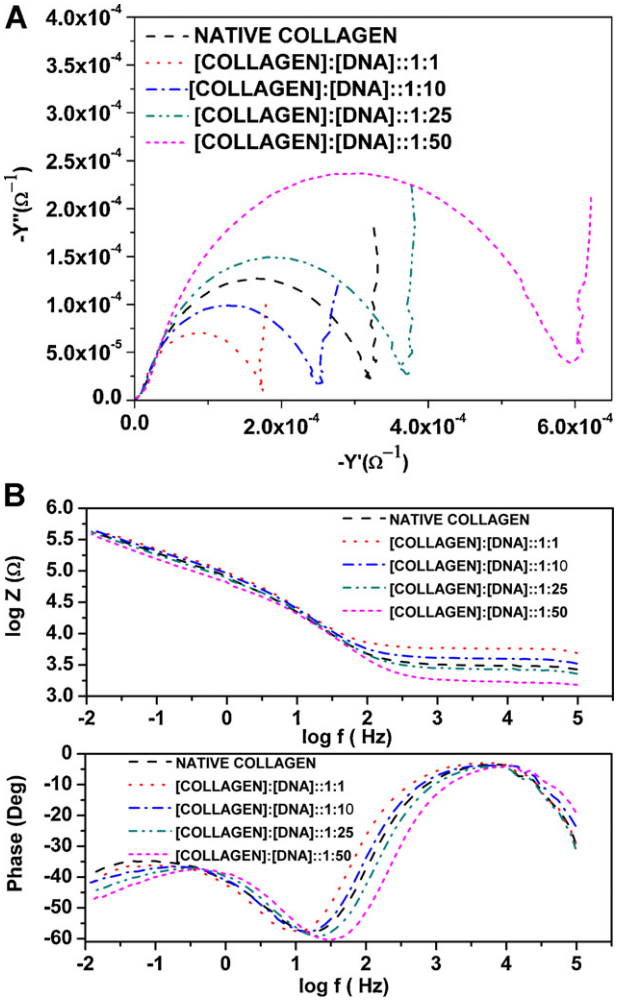

Fig. 3. (a) Nyquist plots: plots of real part Y' (Ω^{-1}) *versus* imaginary part Y'' (Ω^{-1}) of Faradaic admittance measurement. (b) Bode plot: plots of log$f$ (Hz) *versus* log Z (Ω) and phase (°).

Table 1
Rpn ratio of native collagen and collagen–DNA conjugate at different temperatures.

Temperature (°C)	Rpn values				
	Native collagen	CODN 1[a]	CODN 2[b]	CODN 3[c]	CODN 4[d]
30	0.126 ± 0.001	0.122 ± 0.002	0.014 ± 0.001	0.111 ± 0.002	0.130 ± 0.002
31	0.124 ± 0.001	0.122 ± 0.001	0.129 ± 0.001	0.112 ± 0.002	0.124 ± 0.001
32	0.123 ± 0.002	0.1197 ± 0.001	0.129 ± 0.0015	0.112 ± 0.001	0.121 ± 0.001
33	0.121 ± 0.0005	0.121 ± 0.001	0.126 ± 0.001	0.110 ± 0.002	0.122 ± 0.001
34	0.120 ± 0.001	0.118 ± 0.0005	0.125 ± 0.0005	0.108 ± 0.001	0.119 ± 0.001
35	0.113 ± 0.001	0.114 ± 0.001	0.120 ± 0.001	0.098 ± 0.002	0.117 ± 0.002
36	0.107 ± 0.002	0.108 ± 0.001	0.117 ± 0.001	0.085 ± 0.0015	0.112 ± 0.002
37	0.096 ± 0.001	0.102 ± 0.002	0.109 ± 0.001	0.069 ± 0.001	0.108 ± 0.001
38	0.090 ± 0.001	0.09 ± 0.001	0.097 ± 0.002	0.054 ± 0.0005	0.095 ± 0.001
39	0.034 ± 0.001	0.027 ± 0.002	0.053 ± 0.002	0.003 ± 0.001	0.059 ± 0.001
40	0	0	0	0	0

[a] CODN 1 – [Collagen]:[DNA]::1:1.
[b] CODN 2 – [Collagen]:[DNA]::1:10.
[c] CODN 3 – [Collagen]:[DNA]::1:25.
[d] CODN 1 – [Collagen]:[DNA]::1:50.

from B to Z form has been observed while collagen is aggregating along the surface of helix of CT–DNA at pH 6 and a conversion to C form at pH of 7 [28]. The viscosity result clearly shows that pH has a great influence on the interaction of collagen and DNA. The increase in the relative viscosity of collagen–DNA conjugate at the pH of 6 and 7 is due to the formation of collagen aggregates (fibrils) that was easily evident by the opacity of sample solution. Collagen is known to self assemble at physiological pH. Well-arranged phosphate groups, also aids in the self assembly process to make ordered aggregates. This indicates that well-arranged phosphate groups of DNA and water layers structure around the DNA as well as collagen accelerate the fibrilogenesis of collagen in aqueous media [9,29].

It could be seen from the CD studies that DNA does not adversely influence the conformational stability of collagen. The triple helical structure of collagen is retained even after interaction with DNA. The temperature dependent CD measurements indicate that there is an influence of DNA on the thermal stability of collagen. It is well known that the melting temperature of native collagen is about 37 °C [9,10]. The stabilization of collagen molecule lower than 37 °C is attributed to the certain factors like space filling models of triple helix with Gly-Pro-Hyp sequence, water mediated hydrogen bond formation, interchain hydrogen bond between the backbone amino and carbonyl group [30]. The physical properties like viscosity, solubility and optical activity of collagen are changed in the complete denaturation process due to the breakage of hydrogen bonds between the adjacent polypeptide chains resulting transition from triple helix to random coil [30–32].

At 40 °C, there is complete denaturation of collagen structure in the collagen–DNA conjugate visualized from the red shift of negative peak and disappearance of the positive peak (220 nm) at that temperature. The Rpn values for native collagen at different temperatures show minor differences from that of the collagen–DNA conjugate at different concentrations. The collagen–DNA conjugate formation was initiated due to the electrostatic interaction as well as hydrogen bonding between the donor group like CH_2, CH (OH) of type I collagen and the acceptor phosphate group of DNA [6,29,32]. The hydrogen bonding are created directly either by collagen(CH)···DNA(PO_4), or by collagen(CH)···H_2O···DNA(PO_4). These interactions could have been affected at higher temperature resulting in the denaturation of protein molecule as well as DNA [29]. Thus, CT–DNA influences the thermal stability of collagen and *vice versa.*

The protein-associated or bound water exhibits dielectric properties due to its hindered rotational mobility. The strength of ion pairs present in between the amino groups and carboxyl groups has an influence on the degree of hydration. Vibration motions within the protein structure and charged side groups of protein contribute to the δ dispersion, especially at the higher levels of hydration, where water appears to act as a molecular plasticizer or lubricant for the protein [33]. Hydration influences the proton formation directly resulting in the variation in the dielectric properties thus changes in the permittivity as well as the admittance of the protein [34].

There is no net permanent dipole moment for DNA due to the presence of helical structure, there is a large dipole moment directed along the axis of double helix [35,36]. Interaction between collagen and DNA stabilizes the hydration shell of DNA. The strength of ion pairs and hydrogen bonds between protein, DNA and water is a strong function of the degree of hydration of the protein molecule. The phosphate groups and water molecules in the minor groove of DNA causes the changes in the torque induced by the applied electric field. This torque tries to rearrange the dipole moment of the polar side chain groups as well as water molecules situated in the hydration shell of the collagen triple helical structure [37].

The charge nature of the collagen is neutralized after conjugation with negatively charged DNA, so that the more dipolar nature of the protein starts to disappear leading to decrease of permittivity (*i.e.*, admittance) at lower molar ratio of collagen:DNA. There is an increment of permittivity of the collagen–DNA conjugate with the increase of DNA concentration, which implies that the negative charge of DNA is now predominant in the conjugate giving rise to a large induced dipole moment.

5. Conclusion

The present investigation provides evidences for the binding of DNA to collagen. Viscosity studies indicate that the collagen–DNA conjugate can be maintained without the formation of collagen fibrils at pH 4 and 5. It is also observed that there is a marginal increment in the thermal stability of collagen–DNA conjugate at different molar concentration ratios. The influence of electrostatic interactions on the hydration behaviour of the collagen–DNA conjugate has been elucidated. For different concentration ratios of collagen–DNA conjugate, there is a change in the ionic behaviour of the conjugate, which rather influences the dielectric behaviour of protein.

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