Impedance Analysis: A Tool for Understanding Changes in Hydration Dynamics of Collagen on Crosslinking

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Abstract

The interaction of collagen with its water of hydration is of special importance in determining the mechanical properties of connective tissues such as cartilage, tendons, and ligaments which is also implicated in associated disorders. In this work, we have investigated the influence of crosslinking phenomenon on the hydration structure of collagen fibers using impedance as a tool. Impedance is a powerful technique for understanding solute-solvent interactions. Admittance measurements also provide powerful insights into the nature of solvent interactions with solute molecules. The crosslinking agents chosen for this study are polyvinyl alcohol (PVA), polycaprolactone and Guar gum (GG). Bode and Nyquist plots reveal that collagen-PVA composites have permitivities in between collagen and PVA which also vary with the concentration and crosslinking. Their tan δ values increase as the frequency decreases which, indicate more dielectric absorption. This shows that crosslinking leads to decrease in charge characteristic of collagen; an increase of admittance have been observed for the collagen-PCL composites indicating changes in polarizability of collagen and local restructuring of water near polar-non polar groups. For collagen-GG composites, decrease in permittivity with increase in GG concentration indicates the alteration of polarizability of functional groups which, in turn destroy the dipolar nature of protein molecule. This explains that the hydration state of the interface changes when protein adsorbs onto the surface or interacts with GG.

1. Introduction

The frequency dependence electrical impedance is informative for the structural and functional behavior of the biological macromolecule. It permits to understand the underlying mechanisms of various cellular functions (Scholz and Anderson 2000). The dielectric response of a material represents a measure for the mobility of the free and bound charges of the macromolecule, number and strength of the electric dipoles (Barnes and Greenebaum 2006). An electric field in the radiofrequency range causes dielectric relaxations to protein solutions due to orientation polarization. It is assumed that biological macromolecules in solution behave as rigid ellipsoids (Hendrickx et al. 1968). The interpretation of the induced dipole moments is based on the orientation of permanent dipoles with respect to the ellipsoidal axes of the macromolecule. The presence of ions interacts with the applied electric field, resulting in the conduction of current and polarization effect at the structural interfaces reflecting the structural and compositional information.

In general, three discrete regions of dispersion i.e. α-dispersion,β-dispersion below at 100 MHz and γ-dispersion at a maximum of 20 GHz can be identified in biological tissues as depicted by Dean et al. (2008). The broad rotational orientation of either the entire macromolecule or of specific groups of protein results the α-dispersion due to counter ionic

diffusion whereas β-dispersion is largely caused by reorganization of "bound" water molecules near the surface of the protein or macromolecule (Marzec and Warchoł 2005). These "bound" waters undergo dispersion at a lower frequency because they are rotationally and translationally hindered, and possess higher and more ordered dipole moments compared with bulk solution (Moisel et al. 2008). Water is strongly dielectric, and absorption of bulk water results γ-dispersion (Pethig 1992).

Collagen is the major constituent of the connective tissues. It is recognized by the characteristic proline-rich domain, where Gly is found in every third residue with tripeptiderepeats of–(Gly-X-Y-)$_n$–in which proline (X) and hydroxyproline (Y) holds 20% amino acid composition. Synthetic biodegradable polymers like highly hydrophilic Poly vinyl alcohol (Lee et al. 2005) with numerous active sites for hydrogen bonding, hydrophobic Poly-ε-Caprolactone (PCL) (Dash and Konkimalla 2012) and natural polysaccharide like guar gum (Manikoth et al. 2012) have gained importance in the emerging area of biomedical research because of their many advantageous properties required for tissue engineering.

The micro and nano-fabrication of collagen based biomaterials lead to variation in physico-chemical characteristics *viz.* hydration dynamics, porosity& density. Molecular mobility, polarization and conductivity on the protein-additives interfaces is affected by the additive concentration as well as the nature of the additive concentration, which influences the hydration shell of the protein molecule. The aim of this study is to elucidate hydration dynamic of collagen based blend systems, which in turn will enhance their various applications.

2. Experimental

2.1. Materials

2.1.1. Isolation of type I soluble collagen

Acid soluble type I collagen was obtained from rat tail tendon using the salting method (Chandrakasan et al. 1976). The stock solution concentration was determined using the method of Woessner (1961) and was stored at 4ºC. The purity of the isolated type I collagen was confirmed by SDS-PAGE.

2.1.2. Other reagents

PVA of molecular weight 1,40,000 (g/mol) was supplied by Himedia. Polycaprolactone (PCL, average M_n ~10,000 by GPC, average M_w ~14,000), guar gum and genipin (M.W. 228 (g/mol), 98% by HPLC) wereprocured from Sigma Chemicals Co., USA. Water used for these studies was of Millipore grade.

2.2. Fabrication of collagen based sample

Composites were prepared by mixing suitable volumes of additive solution (PVA and guar gum dissolved in aqueous solution, PCL dissolved in glacial acetic acid) and collagen (0.5 μM) into acetate buffer of pH 4.2 in different molar ratios under stirring condition for 2 h at 4

^{o}C . The molar ratios of collagen/PVA used were 1:3, 1:1 and 3:1. Genipin of 0.01% on weight of collagen was added for stiffening the collagen network. The samples were further stirred for 3 h at 4 ^{o}C. The collagen-PCL molar ratio were 1:1, 1:2, 1:3 and 1:4 and collagen-Guar gum molar ratio were maintained at1:1, 1:2, 1:3, 1:4 and 1:5.

2.3. Characterization of electrical behavior at the Interfaces

AC impedance analysis was carried out to determine the electrical behavior of the composite systems by means of CH Instrumental (U.S.A.) electrochemical analyser CH-model 660B. The measurement set-up consisted of 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 operating conditions were Init E (V) = 0.09, high Frequency (Hz) = 1e+5, low Frequency (Hz) = 0.01, amplitude (V) =0.005, quiet Time (s) = 2, cycles (0.1-1Hz) =1, cycles (0.01-0.1Hz) = 1, cycles (0.001-0.01Hz) =1. All experiments were done in triplicates and average values are reported.

3. Result and discussion

The molecular asymmetry, polar nature and the capacity to form hydrogen bond are the key factors for their unique physical properties (Marzec and Warchol 2005). The hydrophobic groups of protein molecule are buried into the interior core whereas the hydrophilic groups reside on the surface of the protein having an affinity to interact with water molecules from its surrounding aqueous environment. The large permanent dipole moment of a protein molecule is caused due to the positive and negative charges resulting from the presence of ionisable acidic or basic amino acid side chains. The permanent dipole moment of a protein depends on the pH of the solution, intra-molecular mobility, molecular conformation as each polar entity generates characteristic response towards an applied electric field. The static permittivity of the protein molecule is higher than the water molecule due to higher polarisability.This frequency-dependent relationship between impedance, conductivity and relative permittivity is given by the expression

$$Z^*(\omega) = Z'(\omega) + jZ''(\omega) \quad (1)$$

Where, Z*(ω) is the total (complex) impedance, Z′(ω) and Z″(ω) are the real and imaginary components of Z*(ω) respectively, ω is the radial frequency. The conductivity (inverse of resistivity) reflects the conduction properties of the biological material.

Admittance (Y*(ω)) is the inverse measurement of impedance. The dissipation factor (D) can be calculated from the relation.

$$D = \tan\delta = Z''(\omega)/Z'(\omega) = \sigma/2\pi f\varepsilon_0\varepsilon_r \quad (2)$$

Where, f is the applied frequency, σ is the electrical conductivity, ε_o is the dielectric permittivity of free space, ε_r is the relative permittivity.

Full hydration is attained when less than half of the surface residues are actually hydrated. In collagen, some water molecules form hydrogen bonds with the hydrophilic residues of the protein and others will experience strong electrostatic interaction with charged residues like glutamate and lysine residues. The water molecules surrounding such hydrophobic groups

will be forced to form networks of hydrogen bonds with each other in a different way from those characteristic of normal, bulk water (Pethig 1992).

Di-electrical behavior of different systems is an indicator of collagen hydration behavior to be exploited for their materials properties. Dielectrical behavior of different collagen-additive systems is plotted in Fig. 1, Fig. 2 and Fig. 3. Bode plot and Nyquist plot of different collagen-additive system show that the dielectric behavior of the composites can be tuned by altering the recipe of composites. The dielectric response of various composites shows that the charge moves through the samples via a process of charge-hopping between discrete sites (Schlag et al. 2000). High probability of charge transfer between two adjacent amino acids results in high coupling strength (Peppas and Sahlin 1989). Bode plot of collagen-PVA systems (Fig. 1 a) indicates that composites have behavior in between that of pure collagen and pure PVA. From the Nyquist plots (Fig. 1 b), it can be seen that collagen has the highest permittivity and PVA has the lowest permittivity. This is owing to the fact collagen is a protein with various functional groups, which leads to its charged behaviour. The collagen-PVA composites have permitivities in between that of collagen and PVA and vary with the concentration and crosslinking. Their tan δ values increase as the frequency decreases as the resistive component of their impedance dominates. Large tan δ indicates that there is more dielectric absorption. At the collagen:PVA ratio of 1:3, crosslinking does not affect the electrical behavior of the composite. However, at 1:1 crosslinking decreases the permittivity and at ratio 3:1, permittivity increases. Increase in permeability results lower impedance. The above results suggest that crosslinking influences the charge characteristic of collagen depending on the concentration of PVA which can be related with the lower molecular mobility due to the crosslinked process.

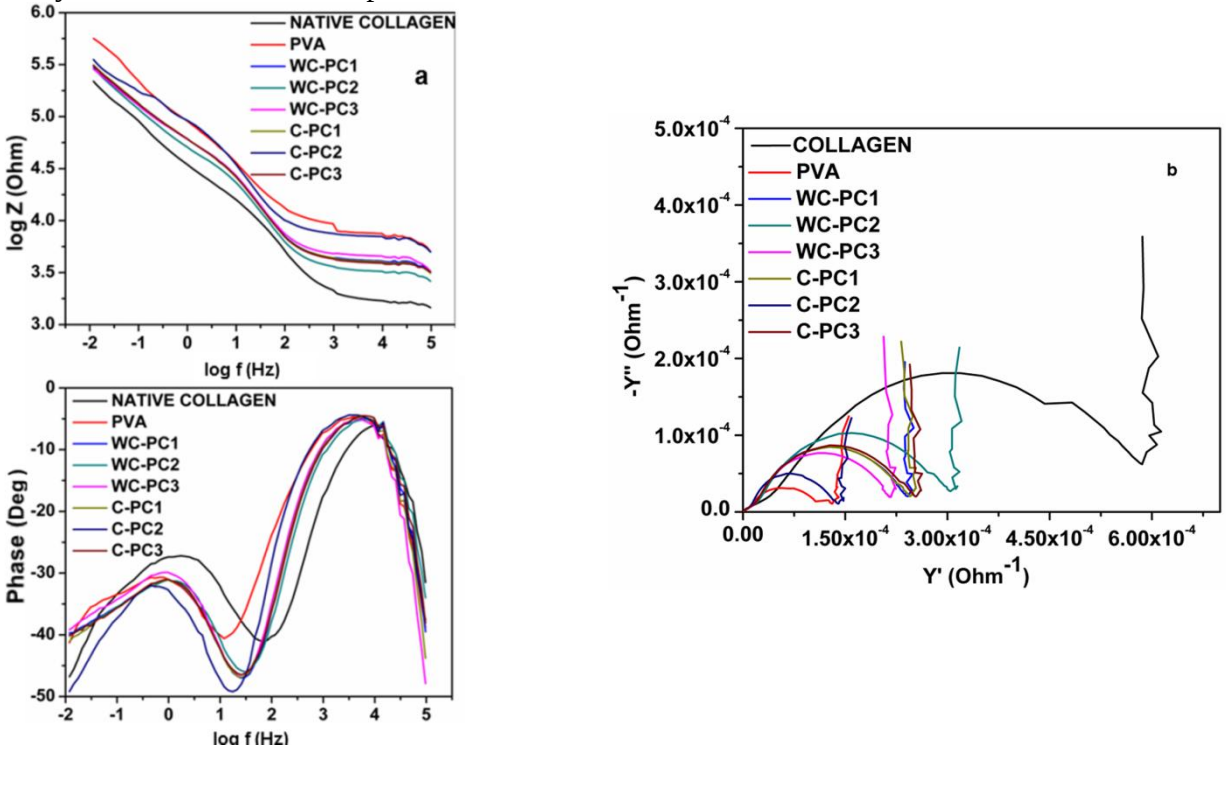

Fig.1. Dielctric behavior of various collagen-PVA systems at 25ºC. (a) Bode Plot: Changes in the log (Z/Ohm) and –Phase(Deg) as a function of log (Freq/Hz). (b) Nyquist plot: Changes in the imaginary values of admittance (Y'', S) as a function of real admittance value (Y', S).

Pure PCL shows the lowest admittance value (shown in Fig. 2 b), due to its covalent nature. The permittivity profile of the composites shows that the permittivity of the system depends upon the concentration of PCL. The dielectric response of PCL is essentially flat, indicating no charge movement and no charge displacements in this frequency range. The permittivity of composites decreases at 1:1 and 1:2 of collagen: PCL ratio compared to native protein. The permittivity of CPCL-3 is high compared to the CPCL-1 system due to the restructuring of water molecules. The permittivity profile of CPCL-4 system shows very lower admittance value compared to CPCL-2. This may be attributed due to the dominant nature of PCL in the composite system. Fig. 2 (c) shows that the resulted coupling strength via charge transfer is maximum for CPCL-3 system. The rate of electron transfer across hydrogen bonds is greatly increased when the electron motions are strongly coupled with those of the protons. Thus the coupling strength between local charged states of protein, responsible for charge transfer sites, was influenced by the concentration of the PCL.

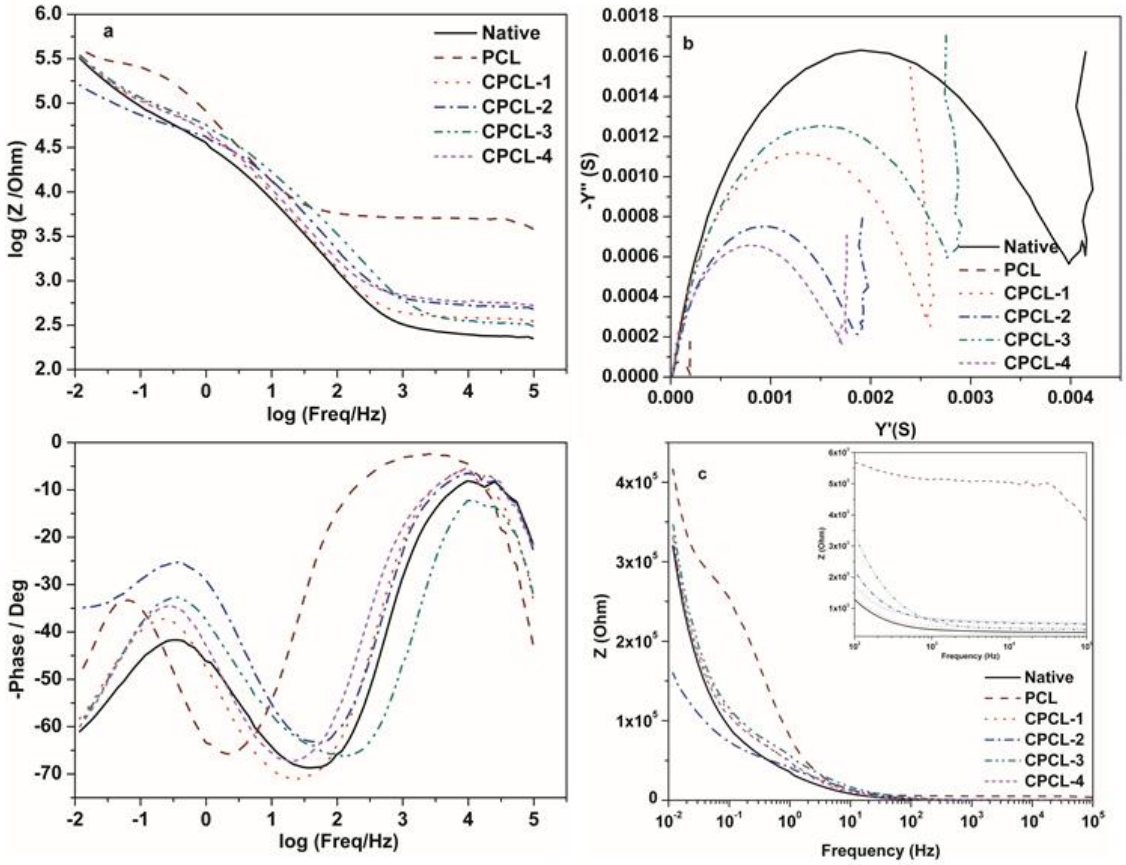

Fig.2. AC impedance analysis of various collagen-PCL composite systems at 25ºC. (a)Bode Plot: Plot of log (Z/Ohm) and –Phase(Deg) as a function of log (Freq/Hz). (b) Nyquist plot: Changes in the imaginary values of admittance (Y", S) as a function of real admittance value (Y', S) of various collagen-PCL composite systems at 25ºC. (c) Maxwell-Wagner dispersion: Profile of dielectric response.

Fig. 3 indicates the dielectric behaviour of collagen-guar gum composites. Decrease inpermittivity (i.e. admittance) with increase of guar gum concentrationindicates the

alteration of polarizability of functional groups which in turn destroy the dipolar nature of protein molecule.

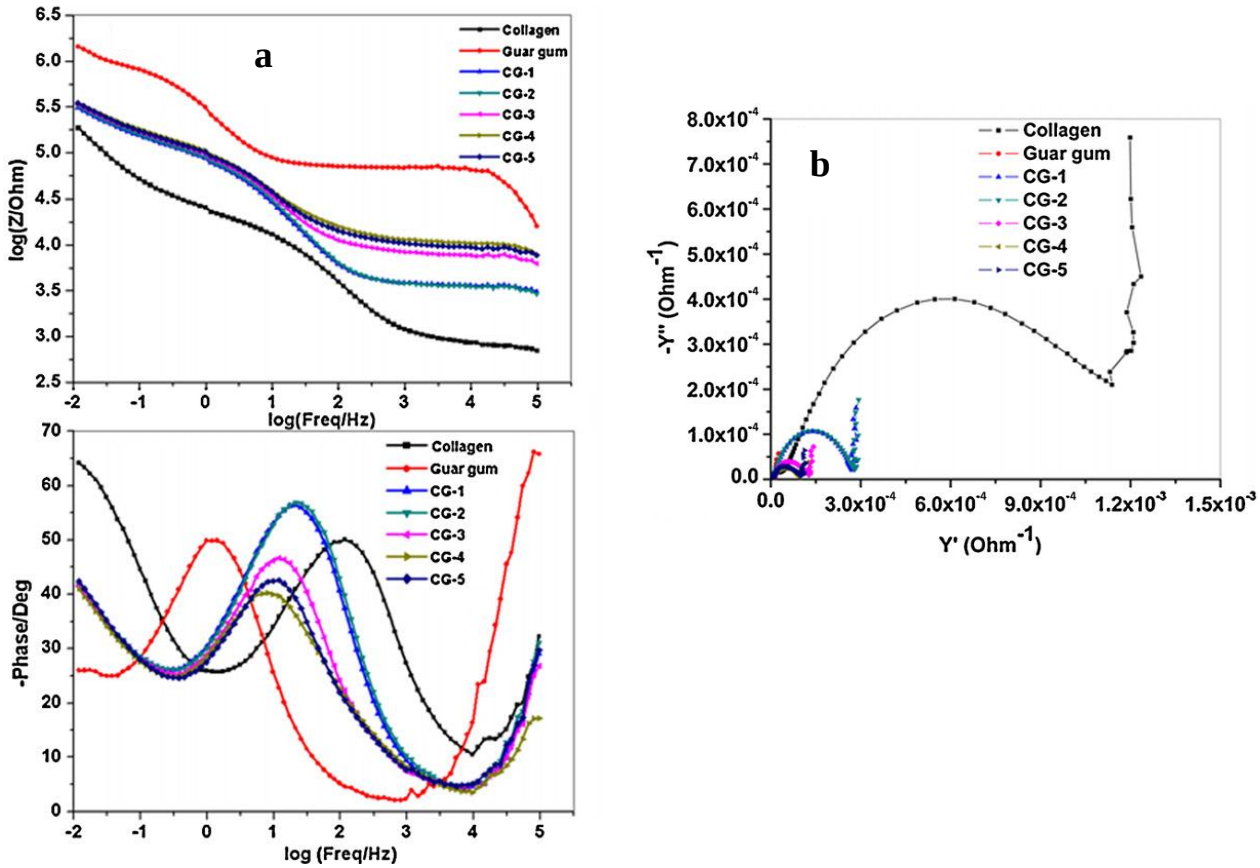

Fig.3. (a) Bode Plot of variuos collagen-Guar gum system: variation of log Z (ω) (Ω) and Phase (Deg) with log f (Hz). (b) Nyquist Plot: –Y' (ω) (Ohm^{-1}) versus –Y'' (ω) (Ohm^{-1}) .

At the pH 4.5, collagen carries the net positive charges on its triple helical structure. The strength of ion pairs present in between the amino groups and carboxylgroups of protein as well as other polar molecules influence the degree of hydration of protein.Although the charge nature of the collagen is unaffected after complexation with non-ionic additives, the hydration shell of the protein molecule is tuned with the additive concentration. The change in dielectrical properties explain that the changes in hydration at the interface when protein interacts with additives.

Conclusion

The polarizability of the collagen is tuned by the additive nature as well as concentration. Ionic charge initiates polarization mechanisms through charge accumulation at structural interfaces. Their dielectric properties reflect contributions to the polarization from both structure and composition of the designed material which is an indicator of collagen hydration behavior to be exploited for their materials properties. Behavioural changes of the rotational as well vibrational motion of the polar functional groups associated with the

protein are due to the formation of hydrogen bond between functional groups of protein and additives. This leads to the alteration of the hydration shell of the collagen. This study highlights that the impedance analysis of composites can be exploited for understanding of the crosslinking of collagen.

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