Role of water content in dielectric properties and delayed luminescence of bovine Achilles' tendon

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Abstract In order to investigate the role of water network in collagen structure, measurement of dielectric permittivity was performed on bovine Achilles' tendon as a function of water content. The data show a sudden decrease of the permittivity at each measured frequency value when the tendon humidity decreases. A similar behaviour is shown by the total number of photons emitted in delayed luminescence (DL) experiments. The comparison of the two results is in agreement with the hypothesis that DL is connected to the excitation and subsequent decay of collective electronic states, whose properties depend on the organized structure of the system.
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1. Introduction

Dielectric spectroscopy (DS) offers important and sometimes unique information on the dynamic and structural properties of substances [1]. It has been used as a tool to investigate the dielectric properties of biological systems for over 75 years and it has provided a great deal of information on structure and properties of biological molecules, cells and tissue. It is especially sensitive to interface polarization and intermolecular (dipole–dipole) interactions, and it is largely used to monitor cooperative processes [2].

The dielectric properties of biological systems have been extensively studied in the past [3–9]. They typically display extremely high dielectric permittivity at low frequency, falling off in distinct steps with increasing frequency. In general the biological materials are characterized by three major dispersion regions for extra low frequencies (α-dispersion), radiofrequencies (β) and microwave frequencies (γ). Different mechanisms account for this behaviour as a function of frequency.

In this work the DS is used in the frequency range 500 Hz to 10 MHz corresponding to the β-dispersion region, that is usually interpreted invoking the polarization of cellular membranes [8]. In particular, it is studied how the dielectric properties of the tendon tissue change as a function of its water content, in order to investigate the role of water network in the structure of the collagen molecules constituting the tendons. Indeed native collagen exists only in hydrated state. Water molecules mediate some networks of intra-chain and inter-chain hydrogen bonds contributing to protein stability and self assembling [10].

Collagen represents the prevalent structural protein in the extra-cellular matrix and possesses a relatively regular structure. The collagen molecules consist of three left-handed α-helical chains wounded into a right-handed helix. The most abundant collagen, type I, is obtained from the sequence $(Gly\text{-}X\text{-}Y)_n$, where the first component is the amino acid Glycine (Gly), and X and Y are in most cases the imino acids Proline (Pro) and Hydroxyproline (Hyp) respectively.

Collagen molecules are readily aggregated into fibrils several triple-helices in diameter, that convey the principal mechanical support and structural organizations for connective tissues such as bone, cartilage and tendon [11–13]. In particular, the tendon has a hierarchical, fractal fibrous structure, in which fibrils are in turn aggregated into fibres. The fibres are mostly aligned in parallel bundles and show a waveform or crimp structure along the tendon axis that allows for the extension of the tendons without actual extension of the collagen fibrils.

The collagenous tissue structure appears to be a promising model system to investigate in great detail the relationships between biological organization and the characteristics of delayed luminescence (DL), the phenomenon consisting of the prolonged ultra-weak emission of optical photons after excitation of the system by illumination [14]. Moreover tendon structure let us to think to a ferroelectric liquid crystal (FLC) behaviour where energy transfer can occur by soliton waves. This is also in agreement with the hypothesis that DL is connected to the excitation and subsequent decay of collective states whose properties depend on the organized structure of the system.

2. Materials and methods

2.1. Tendons preparation

The tendons were provided by the slaughterhouse, immediately after the animals were slaughtered. Tendons were stripped of the external sheath and washed in bidistilled water. After washing, the tendons were cut into pieces, which were rinsed in bidistilled water and immersed in a 1 M solution of NaBr for 4 h. Then they were immersed in ether for 2 h, washed in four changes of bidistilled water, dipped in bidistilled water and stored at low temperature (about 11 °C) . Using this procedure, a simplified structure composed only by collagen chains and bounded water is obtained.

To perform the measurement the pieces are cut into slices perpendicular to the long axis of about 1 mm thick and 10 mm diameter.

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2.2. Dielectric spectroscopy measurements

The dielectric measurements were performed using an automatic swept-frequency impedance analyser (AGILENT 4294A coupled with the AGILENT 16451B probe).

In principle, the dielectric properties (permittivity ε and conductivity σ) of a sample could be calculated from measurements of the complex impedance of the probe in air and of the unknown sample, using the following relationships:

$$\varepsilon = c/c_0, \quad \sigma = G/K$$

where c and G are the sample capacitance and conductance, c_0 is the air capacitance and K is a geometrical factor. In practice, the measure of conductive materials is not so straightforward because of some parasitic effects. To avoid systematic errors a careful calibration was performed. Moreover, to take into account the electrode polarization effects, all the data presented in this work have been corrected using an additional measurement of a ionic solution which was equivalent in conductivity to the sample, in accordance to the method used in [9]. As a test some measures were performed on standard samples and their known values of permittivity were reproduced. The auto-consistence of experimental results was also checked by the Kramer's Kronig relations and by inspecting the Cole–Cole plots.

The measurement technique and associated instrumentation used in this study give a random reproducibility of about 0.1% across the used frequency range, for measures on standard samples of uniform composition. Biological tissues are inhomogeneous and show considerable variability in structure and composition. For this reason many samples (more than 15) have been measured to evaluate the uncertain of the measure at different frequency values. Three runs for each sample were performed and the average value was determined. The spread of the data with respect to their average value is about 20% for frequencies less than 1 kHz and about 5% for higher frequencies.

2.3. Delayed luminescence measurements

The experimental set up [14] is able to measure, in single photon counting mode, photons in the range of wavelengths from 200 to 850 nm. A pulsed nitrogen laser (LASERPHOTONIC LN203C) (duration ≈5 ns, λ = 337.1 nm) is used as light source, and after each illumination, the radiation is detected by a low-noise photomultiplier (THORN EMI 9558 QA), cooled to −20 °C to decrease the dark current. Photon counts are stored by a channel scaler, using a dwell time optimized to measure the decay dynamics. The background emission was measured and subtracted from the measurements of each sample, so that the time trend and the total number of photons emitted during the recording time could be obtained.

3. Results

The typical plot of permittivity ε and conductivity σ of native tendon as a function of the frequency is given in Fig. 1. The measured data are in agreement with previous results [9].

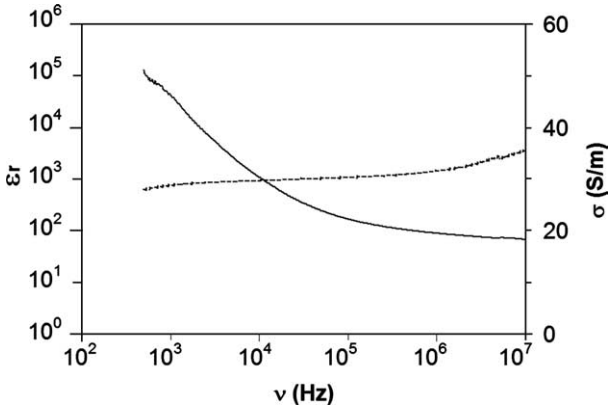

Fig. 1. Dielectric permittivity (solid line) and conductivity (dashed line) of native (full hydrated) tendon as a function of the frequency.

The dielectric permittivity of natives samples was measured as a function of the time elapsed from tendon excision in the studied frequency range. The measures were performed on the same samples that were dipped in bidistilled water and stored at a temperature of 11 °C after each measurement. The data for different values of frequency are reported in Fig. 2 and shown a decrease of the dielectric permittivity of about one or two order of magnitude in the first three days from the excision time, towards a stationary value. The measures reported in the following have been performed three days after the slaughter, i.e., when the tendon permittivity does not change more.

The samples were dehydrated gently at room temperature. During drying, samples were kept flat by putting them between rigid plastic plates covered by filter paper and gently compressed (pressure less than 2×10^4 N/m^2). A suitable holder system was designed to improve the natural convection of surrounding air, allowing to reduce the inhomogeneity of the material during the drying process.

In Fig. 3 the dielectric permittivity at different frequencies is reported as a function of the tendons humidity, calculated using the ratio:

$$h = \frac{W - W_{\text{dry}}}{W_{\text{dry}}}$$

where W is the weight of the sample during the drying procedure and W_{dry} is the weight of the fully dried sample (when the weight does not change more). The quantity h gives than the ratio between the mass of the water content and the mass of the dry collagen.

The behaviour of dielectric permittivity as a function of the humidity can be divided into three regions.

For $h > 0.5$ the dielectric permittivity is independent from the water content, while it depends strongly on the frequency, varying of about 4 order of magnitude from 500 Hz up to 10 MHz. At higher frequencies the dielectric permittivity is mostly equal to the water value. At lower frequencies it became very large.

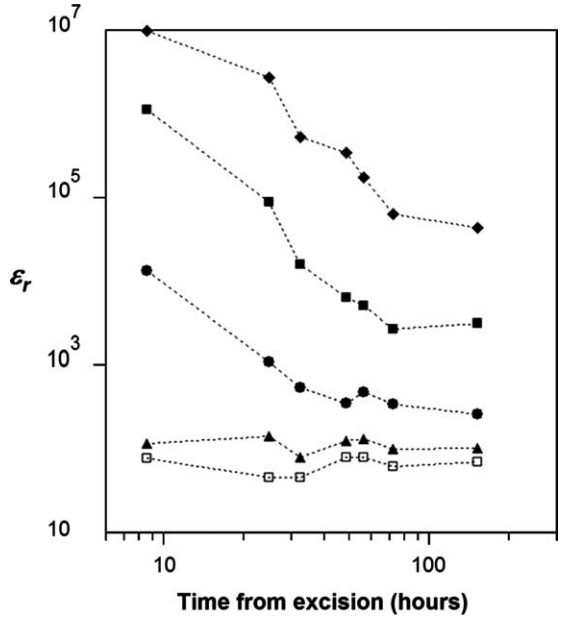

Fig. 2. Dielectric permittivity as a function of the time elapsed from excision for different values of the frequency (500 Hz – ◆, 5 kHz – ■, 50 kHz – ●, 500 kHz – □, 10 MHz – ▲).

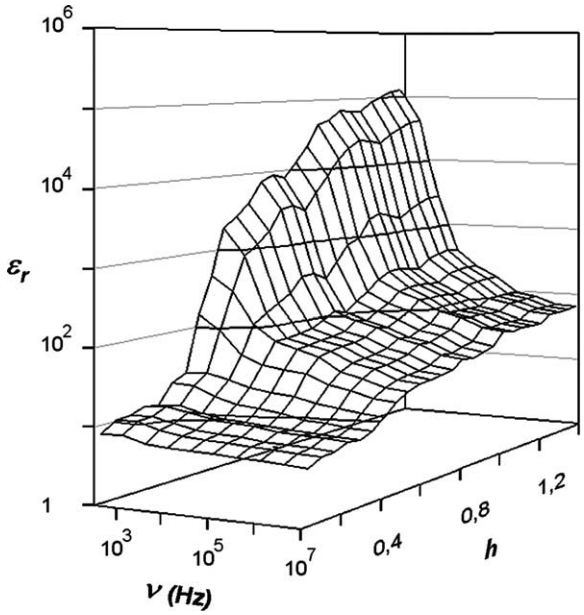

Fig. 3. Dielectric permittivity as a function of the collagen humidity h for different values of the frequency ν.

For $0.1 < h < 0.5$ the dielectric permittivity suddenly decrease. The transition is more evident for lower frequencies.

For $h < 0.1$ the dielectric permittivity smoothly changes. It has the same value for all the frequencies under study and it is independent from the time elapsed from the slaughter (data not shown).

The same behaviour showed by the permittivity values as a function of the humidity is also present in previous measures of DL: it was found that a drastic change in DL kinetics occurred from the native state to the completely dried state [14]. In Fig. 4 the total number of emitted photons (in arbitrary units) and the inverse of permittivity (measured at frequency equal to 5 kHz) are reported as a function of the humidity of the tendon's sample.

Both the dielectric permittivity and the number of DL photons have a constant value as a function of the humidity for $h > 0.5$, while they suddenly change at the same threshold humidity value.

These results confirm the fundamental role of hydration in the triple helix structure and are also in agreement with molecular dynamics calculations [15]. Recent measures of synchrotron-radiation diffraction pattern have also shown that the water molecules surrounding the collagen triple helix can be grouped in two equally populated shells [16]. In the first shell the water molecules are directly bound to the carbonyl groups and they act as anchoring points for other water molecules, that occupy the second shell. The data reported in Fig. 4 show that both the dielectric permittivity and the DL emission do not change if the second hydration shell is completely dried, but they suddenly change if water molecules directly bounded to the polypeptide chain are removed.

4. Discussion

The correspondence between the behaviour of dielectric permittivity and DL photon emission as a function of tendon water content can be set in the framework of the novel hypothesis that the self-assembly of collagen rich tissues is determined by liquid crystal-like flow processes.

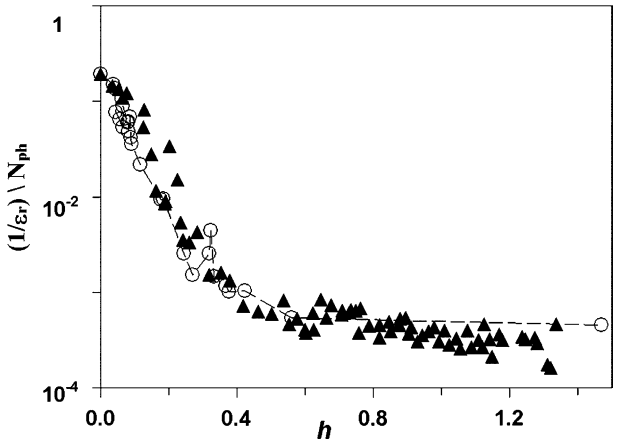

Fig. 4. Comparison of DL and DS measurements as a function of the tendon humidity: (○) total number of DL photons emitted (dashed line is used as eyeguide) in arbitrary units, (▲) inverse of dielectric permittivity at 5 kHz.

Polarized light microscopy has shown that in native tendons fibrils are parallel or antiparallel but subject to an undulating waviness or crimping on a scale of several micrometers [13]. Such crimp let us to think to a tilted smectic arrangement of a liquid crystal, which exhibits ferroelectricity when it is composed of chiral molecules [17], as collagen molecules. Due to their molecular structure, FLCs present a spontaneous helixing of the polarization, so that over macroscopic distances the polarization averages to zero. However, if they are subjected to an external electric field, they show their ferroelectric properties, as high dielectric permittivity values.

In addition, theoretical calculations based on the simple visco-elastic equations suggest the possibility of electrical solitary wave propagation in FLC under proper boundary conditions [18,19].

Recently, a correlated solitons model has been developed in order to describe both qualitatively and quantitatively the main features of the DL from biosystems [20,21] and it has been successfully applied to reproduce the DL data of the algae *Acetabularia acetabulum* [21,22]. The possibility to describe the native tendon as a ferroelectric liquid crystal-like system supports the idea of a possible existence of auto-localized electron states in the triple helical conformation of collagen with lateral inter-chain hydrogen bonds and in presence of hydrogen bonds to water molecules.

From the point of view of the crystal structure we can model each tri-peptide (Gly-X-Y) as a unit cell of a one-dimensional lattice, whose electronic structure is similar to semiconductors, with the filled valence band and the empty conduction band separated by a energy gap. The DL is connected to formation and dissociation of auto-localized electron states, whose energy level is in the forbidden band. The ground state of such one-dimensional lattice, described by a like-Holsten Hamiltonian and studied in the frame of a variational approach, can be represented by one of the three quasi-particle states: almost free electron, soliton and small polaron. The occurrence of the different types of quasi-particle states depends on the relative relation between the three characteristic energies of the system

(that is the energy band of a free electron, the Debye energy and the polaron binding energy). A phase diagram for the different types of quasi-particle state can be determined analogously to what reported in [23,24]. According to primary calculations (a complete work will be published shortly), on decreasing the dielectric permittivity, which in our experimental results happens on drying the sample, the ground state in the phase diagram moves from a soliton state to a small polaron state, giving rise to different DL kinetics regimes. This can take into account for the increase of the total number of emitted photons on drying sample (see Fig. 4). Worth to mention, small polaron formation in dry DNA was also investigated to explain the phenomenon of charge transport [25].

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