Glassy state of native collagen fibril?

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Abstract – Our micromechanical experiments show that viscoelastic features of type-I collagen fibril at physiological temperatures display essential dependence on the frequency and speed of heating. For temperatures of 20–30 °C the internal friction has a sharp maximum for a frequency less than 2 kHz. Upon heating the internal friction displays a peak at a temperature \( T_{\text{soft}}(v) \) that essentially depends on the speed of heating \( v \): \( T_{\text{soft}} \approx 70 \) °C for \( v = 1 \) °C/min, and \( T_{\text{soft}} \approx 25 \) °C for \( v = 0.1 \) °C/min. At the same temperature \( T_{\text{soft}}(v) \) Young’s modulus passes through a minimum. All these effects are specific for the native state of the fibril and disappear after heat-denaturation. Taken together with the known facts that the fibril is axially ordered as quasicrystal, but disordered laterally, we interpret our findings as indications of a glassy state, where \( T_{\text{soft}} \) is the softening transition.

Connective tissues (tendon, chord, skin, bones, cornea and dentine) are complex, hierarchical structures, which have widely different mechanical and biochemical demands with respect to strength, elasticity and energy storage [1]. These demands are met via adaptation of the tissue’s hierarchical structure. Its major building block is the fibril made from triple-helical type-I collagen (macro)molecules, the most abundant protein in mammals [1]. The fibril combines axially ordered quasicrystalline structure with lateral disorder [2,3]. Both these aspects have been actively studied in the last decades [1–5], but many issues are still open. This subject is relevant for medicine, since a number of diseases (e.g., arthritis) relate to abnormalities in the fibril structure [1].

Here we shall study viscoelastic features of the native type-I collagen fibril. Our experimental results point out the existence of a glassy state at physiological temperatures. This state is displayed via frequency-dependent viscoelastic characteristics (Young’s modulus and the damping decrement) of the native fibril. Upon heating the fibril goes out of the glassy state, an effect known as the softening transition [6,7]. The temperature of this transition depends essentially on the speed of heating.

We confirmed that glassy features are not seen for heat-denatured fibril. This will be the first example of a room temperature biopolymer glassiness, because the glass transition in globular proteins was experimentally observed at \( \approx 200 \) K [8–13]. Several methods contributed to the understanding of this transition: micro-mechanical experiments [8], NMR [9], Moessbauer spectroscopy [10], calorimetric studies [11], and X-ray scattering of synchrotron radiation [12]; see [13] for reviews. It is believed that the large-scale conformational motion of proteins freezes at \( \approx 200 \) K, analogously to freezing of cooperative motion in glass-forming liquids [13] and segmental motion in synthetic polymers [6,7].

The fibril’s structure. – In rat tail tendon the fibril consists of \( \approx 10^3 \) triple-helical collagen molecules of diameter 1 nm and length 300 nm staggered together. We conventionally separate the collagen molecule into 5 segments [2]. The segments from 1 to 4 have equal length \( D = 67 \) nm (this value of \( D \) is slightly tissue dependent), segment 5 is of length 0.5 \( D \). The fibril is ordered along its axis: nearby molecules overlap by distance \((0.5 + k)D\), where \( k = 0, \ldots, 4 \) can assume any value between 0 and 4. This is a quasicrystalline order, because the long-range order is displayed without strict translational symmetry, and the four possible types of overlaps are distributed along the fibril. The most frequent overlap is 0.5 \( D \) [14]. It

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takes place between segments 1 and 5 and leads to crosslinking between the two molecules via terminal telopeptides located at those segments [2]. All other values of the overlap led to weaker types of bonding (hydrophobic and van der Waals interaction). Laterally (perpendicular to the axis) the fibril contains much less structure; in type-I collagen fibrils there are indications of quasi-hexagonal order [4]. There are also experimental results on the supramolecular (microfibrillar) structure in the fibril [2,3]. This information was obtained mainly from X-ray diffraction experiments that however show a significant amount of diffuse scatter indicating that the fibril contains disordered regions, especially in the gaps between the molecules [2,3]. The lateral disorder led some authors to liken the fibril structure to smectic liquid crystal [5]. So far no direct experiment indicated the existence of such a structure, e.g., no first-order phase transitions were seen between disordered and smectic/nematic phases; such transitions are common for synthetic liquid crystalline polymers [7]. A combination of crystalline order and disorder is not unusual for solid-state biopolymers, e.g., micromechanical experiments that uncovered the low-temperature glass transition in globular proteins were carried out on a crystal, where each site contains one globular protein in glassy state [8].

Materials and methods. – Collagen fibril samples with diameter starting from 1 µm and the length 1–0.1 mm were extracted from Achilles tendons of young rats by means of shaking and pulling using micro-tweezers in 96% ethanol at a temperature of 5°C. We call our sample fibril, because its diameter is closer to the accepted diameter of the fibril than to that of the fiber. Indeed, the fibril diameter established via electron microscopy varies between 40 nm and 0.5 µm [1]. For real fibrils this value is underestimated as the electron microscopy demands drying of the collagen samples.

The sample under investigation was enclosed in the experimental chamber and placed in a temperature-controlled cabinet with the temperature maintained at 25°C. The sample was allowed to equilibrate at a given humidity for several hours. Relative humidities from 97 to 32% in the chamber were achieved by means of CaCl₂ solutions of different concentrations, while the relative humidities of 15% and 10% were obtained via saturated solutions of ZnCl₂ and LiCl, respectively.

Young’s modulus $E$ and the logarithmic damping decrement $\theta$ were measured via electrically excited transverse resonance vibrations of the sample (fibril cylinder), which is cantilevered from one edge (another edge is free) [8]. $E$ characterizes the elasticity and is defined as the ratio of pressure over strain [6,7]; $\theta$ is determined from the amplitude of the sample oscillations and is defined as the ratio of the energy dissipated during the externally forced oscillations to the energy (reversibly) stored in the sample [6,7]. A large $\theta$ is typical for viscous liquids, while a small $\theta$ characterizes elastic solids.

For measuring $E$ and the amplitude-frequency characteristics of oscillations (employed for obtaining $\theta$), it is necessary to smoothly change the frequency $f$ of the induced oscillations and determine the resonance frequency $\omega$, which corresponds to the maximal oscillation amplitude of the sample’s free end. Young’s modulus of the sample’s main axis is calculated via [15]

$$E = 3.19 \cdot \omega^2 \cdot L^4 \cdot \rho \cdot P/I_{\text{min}},$$

where $L$ is the sample length, $P$ is the cross-section area, $\rho$ is the density, and $I_{\text{min}}$ is the main inertia moment of that section, which corresponds to the deformation plane with the minimal stiffness. For the round cross-section of our samples $I_{\text{min}} = \pi \cdot D^4/64$ [15] and $P = \pi \cdot D^2/4$, where $D$ is the sample diameter (measured with precision 0.02 µm). Thus Young’s modulus is obtained from (1), where $L$, $\rho$, $P$ and $I_{\text{min}}$ are the known sample parameters and $\omega$ is measured during the experiment. Studying samples with different length $L$, one can explore a range of (resonance) frequencies.

Below we compare the native features of collagen fibril with those of the globular protein lysozyme (in solid state). Tetragonal ($P4_32_12$) lysozyme crystals were grown following the method of [16] from a solution containing 3% of lysozyme, 5% of NaCl, 0.2 M of natrium-acetate buffer, and with pH = 4.7. Crystals were fixated for 10 days via diffusion through the gas phase of glutaraldehyde. For another 10 days the crystals were fixated directly into 5% glutaraldehyde solution with the same buffer. Fixed crystals have the parameters of the elementary units ($a = b = 79.4$ Å and $c = 37.6$ Å) close to the native ones: $a = b = 79.1$ Å and $c = 37.9$ Å [17].

Mechanical glassy state. – Glass is a metastable state of matter, which is caught in the process of (very) slow relaxation to equilibrium [6,7,18]. Features of this state are sensitive to observation times (frequencies). It is typically displayed whenever the system does not have enough time to follow the external changes, e.g., when cooling with a small, but finite rate, or forcing the system with a finite frequency. These are, respectively, structural and mechanical glassiness [6,7].

During the mechanical glass transition the system is forced by an external field at a frequency $\omega$ (for us this is the resonance frequency of the sample vibration). When changing $\omega$ at a fixed temperature $T$ (this is achieved by changing the sample length), the damping decrement $\theta(\omega)$ —which characterizes internal friction and thus energy dissipation— displays a maximum at some frequency $\omega_g$ [6,7], Approximately at the same frequency range $\omega \approx \omega_g$, Young’s modulus $E(\omega)$ (or some other elastic modulus) displays a crossover between a relatively large high-frequency value $E_1$ to a relatively small, low-frequency value $E_\infty$. At the glass transition frequency $\omega_g$ (one of) the relaxation time(s) of the systems becomes of order of $1/\omega_g$ [6,7]. In structural glass-formers the maximum of internal friction corresponds to a large
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Fig. 1: (Colour on-line) The logarithmic damping decrement $\theta$ of the collagen fibril vs. the frequency at $T = 25^\circ C$ and varying humidity $A$.

Fig. 2: (Colour on-line) Young’s modulus $E$ of the collagen fibril vs. the frequency at $T = 25^\circ C$ and varying humidity.

viscosity, while two characteristic values of $E$, $E_+$ and $E_-$ refer to, respectively, solid-like and liquid-like elasticity features. The same behavior of $E$ and $\theta$ is seen for a fixed frequency $\omega$ and the temperature changing at a small, but finite speed. The glass transition now refers to the relaxation time being of the same order as the inverse dimensionless temperature speed $[6,7]$. 

Results and discussion. – Figure 1 displays the damping decrement $\theta$ of the collagen fibril vs. frequency $\omega$ and at varying humidity and room temperature of $25^\circ C$. $\theta$ does not depend on the humidity and has a maximum at frequencies lower than 2 kHz. The plateau behavior of Young’s modulus $E$ at the same temperature $25^\circ C$ is seen on fig. 2. The plateau is most clearly visible at the lowest studied humidity, though it is identifiable for the highest humidity as well; see fig. 2.

The results presented in figs. 1 and 2 imply that the room temperature collagen fibril displays glassy features at room temperature. The transition frequency $\omega_g$ is smaller than 2 kHz at $T = 25^\circ C$. Changing the humidity does not alter $\omega_g$, in contrast to the low-temperature glassy features of globular proteins, where the hydration level is known to be essential [13]. For the collagen fibril only the upper value $E_+$ of Young’s modulus decreases (the fibril becomes more flexible) for a higher humidity.

Note that we only identified, but did not fully resolve the peak of $\theta$ as a function of $\omega$. This is because the present micromechanical method does not allow to explore frequencies (much) lower than 1 kHz. One needs, to this end, different methods that we are currently investigating.

To make the comparison with globular proteins even more visible we studied viscoelastic features of crystalline lysozyme at room temperature (each site of the tetragonal crystal contains one globular lysozyme protein). Results presented in figs. 3 and 4 show no indications of glassy behavior. Also, for the native lysozyme the dependence on humidity is essential.

It is expected that upon heating the fibril will go out of the glassy state. For synthetic polymers this phenomenon is known as the softening transition [6,7]; this transition
The heating starts at $19^\circ C$ and the relative humidity $A = 93\%$. is accompanied by the same effects as the glass transition: a peak of the internal friction and a relatively abrupt change of Young’s modulus. Figures 5, 6 show that when heating at speed $1^\circ C$/min the collagen fibril undergoes softening transition at temperatures around $T_{\text{soft}} = 70^\circ C$, where $\theta$ has a sharp maximum. For a smaller speed $0.1^\circ C$/min, $T_{\text{soft}}$ should be also smaller than $70^\circ C$, because for slower heating a lower temperature suffices for displaying the relevant motion within the observation times [7]. Indeed, as figs. 5, 6 show, $T_{\text{soft}} \approx 25^\circ C$. This is the physiological temperature regime for the native collagen. Experiments with different heating rates were carried out on different, but identically prepared samples to avoid irreversibility effects due to heating. This samples have slightly different initial characteristics; this is why figs. 5, 6 present normalized quantities.

These findings can be compared with the glass transition in a synthetic polymer, e.g., rosin [7]. A rosin sample is externally deformed with frequency $2.8$ kHz. Upon heating at speed $1^\circ C$/min, the derivative of the rosin elastic modulus with respect to temperature experiences a jump around $T_{\text{soft}} = 303$ K. This is related to the softening transition from the low-temperature glass phase to the high-temperature viscous-flow state of rosin [7]. The peak of the damping decrement (internal friction) takes place at larger temperature $T'_{\text{soft}} \approx 337$ K. The difference between $T_{\text{soft}}$ and $T'_{\text{soft}}$ decreases for smaller heating speed, but one always has $T_{\text{soft}} < T'_{\text{soft}}$. The same aspect is seen in our figs. 5, 6, where the peak of the internal friction takes place at a somewhat higher temperature than the minimum of Young’s modulus $E$.

We note however that the behavior of Young’s modulus $E$ is more intricate [19] than during the softening transitions in synthetic polymers, e.g., rosin [6,7]. It is seen that for $T < T_{\text{soft}}$, $E$ decreases upon increasing $T$; this is a typical scenario for the softening transition. However, for $T_{\text{soft}} < T$ Young’s modulus starts to increase with temperature. This effect was found in ref. [19] and was related to the formation of additional bonds between collagen molecules. Put differently, the higher-temperature phase of the collagen fibril is not properly viscous flow. A similar pattern of softening transition with a non-monotonous change of the elastic modulus and a peak of internal friction is observed for molecular crystals [20]. Here, however, the relevant frequencies are quite different, since the substance is low-molecular (its molecules are not macroscopic).

Let us see how the softening transition relates to the other characteristic temperatures of the collagen fibril. The denaturation temperature of collagen tendon, as measured calorimetrically, is also located around $60-70^\circ C$ when heating under a speed larger than $1^\circ C$/min [21–24]. The denaturation temperature of a separate rat tail tendon collagen triple helix was determined calorimetrically to be $\approx 38^\circ C$ [25]. This temperature must be close to the equilibrium denaturation temperature, since the heating speed in [25] was rather low, $0.004^\circ C$/min. The single-helix denaturation temperature for heating with $0.1^\circ C$/min is $\approx 38^\circ C$ [25]. Since we find $T_{\text{soft}} \approx 25^\circ C$ for $0.1^\circ C$/min, our observations cannot be explained by tendon or triple-helix denaturation, though these processes can contribute to the effects.

Note that we did not observe any glassy feature for the heat-denaturated collagen fibril, which is prepared by keeping the native sample at $120^\circ C$ for several hours. Calorimetric studies indicated the presence of certain glassy feature in the heat-denaturated collagen tendon [22,24]. These features were very sensitive to hydration changes, and they tended to disappear after annealing the heat-denaturated collagen tendons [24]. In contrast, we found glassy features only for the native state, and observed that the hydration is not essential for these features, e.g., figs. 1, 2 show that changing the humidity does not influence the characteristic glassy frequency $\omega_g$.

Another important issue is whether the low temperature (glassy) of the collagen fibril demonstrates features of
(physical) aging, i.e., is there any dependence of the viscoelastic features on the storage (waiting) times of the native sample? The aging phenomenon is typical for synthetic glassy polymers (hence it is of definite technological importance for these substances) [6,7]. For the native collagen fibril we did not note any sign of aging within storage times of six months; this was the maximal storage time we employed. It is our current opinion that the physical aging times of the native collagen can be comparable with the times of its biological aging (i.e., several years). Likewise, we did not find for the collagen fibril clear indications of the time-temperature superposition [6], which is frequently seen for sufficiently simple synthetic glassy polymers. This can mean two things: either our data for different frequencies $\omega$ (or for different cooling speeds) is not enough to identify such a superposition, or —which is more likely— the time-temperature superposition principle is absent, because the collagen fibril is a complicated, heterogeneous material.

Conclusion. — We close by repeating the main message of the present work: type-I collagen fibril is glassy at its physiological temperatures. The phenomenon is not sensitive to hydration changes, is specific to the native state of the fibril, and constitutes the first example of biopolymer glassiness at physiological temperatures. It may be of relevance for understanding the genesis of collagen-based structures. As we recalled above, the collagen fibril combines lateral disorder with axial quasicyrystaline order. The latter is imposed during the fibrillogenesis and may have kinetic, metastable nature, as is frequently the case with quasicrystals. Both metastable quasicyrystaline structure and lateral disorder may become related to the observed glassy features. To decide on this, one needs further experimental results that, in particular, should clarify the situation for frequencies lower than 1 kHz.

It is of interest to know why collagen fibers need to be glassy in vivo. Here we try to give a plausible explanation. A viable biological system should remain in a non-equilibrium state so that biological molecules can properly perform their biological functions. It is well known that glasses have very slow relaxation to an equilibrium state and thus can remain in a non-equilibrium state for very very long time [6,7,18]. Thus glassy collagen fibers help the biological system to remain in a non-equilibrium state so that the system can be viable.

It is interesting to ask whether a non-equilibrium glassy state of the collagen fibril is needed for its proper functioning. Can it be related to a long-time viability of biological organisms that necessarily ought to be out of equilibrium? A vivid example of this is the ability of ancient seeds to remain viable during several hundred years [26]. In this context we mention a recent series of articles by Ma and Hu [27–31]. They used molecular dynamics to study the relaxation and aggregation of polymer chains; the neighboring monomers along a polymer chain are connected by rigid bonds [27,29]. A polymer chain has a bending-angle–dependent potential of strength $K_b$ and a torsion-angle–dependent potential of strength $K_t$. In [27], they used $K_b = K_t = 0.1$ and found that polymer chains have very slow relaxation as in the case of the lattice spin glass at low temperatures [18]. Ma and Hu also found that polymer chains tend to aggregate as $K_b$ and $K_t$ become very small [29,30]. The approach by Ma and Hu will be useful for modeling aggregation of collagen molecules in the fibril.

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