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Viscoelastic properties of model segments of collagen molecules

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ABSTRACT

Collagen is the prime construction material in vertebrate biology, determining the mechanical behavior of connective tissues such as tendon, bone and skin. Despite extensive efforts in the investigation of the origin of collagen unique mechanical properties, a deep understanding of the relationship between molecular structure and mechanical properties remains elusive, hindered by the complex hierarchical structure of collagen-based tissues. In particular, although extensive studies of viscoelastic properties have been pursued at the macroscopic (fiber/tissue) level, fewer investigations have been performed at the smaller scales, including in particular collagen molecules and fibrils. These scales are, however, important for a complete understanding of the role of collagen as an important constituent in the extracellular matrix. Here, using an atomistic modeling approach, we perform in silico creep tests of a collagen-like peptide, monitoring the strain-time response for different values of applied external load. The results show that individual collagen molecules exhibit a nonlinear viscoelastic behavior, with a Young's modulus increasing from 6 to 16 GPa (for strains up to 20%), a viscosity of $3.84. \pm 0.38$ Pa·s, and a relaxation time in the range of 0.24–0.64 ns. The single molecule viscosity, for the first time reported here, is several orders of magnitude lower than the viscosity found for larger-scale single collagen fibrils, suggesting that the viscous behavior of collagen fibrils and fibers involves additional mechanisms, such as molecular sliding between collagen molecules within the fibril or the effect of relaxation of larger volumes of solvent. Based on our molecular modeling results we propose a simple structural model that describes collagen tissue as a hierarchical structure, providing a bottom-up description of elastic and viscous properties form the properties of the tissue basic building blocks.

1. Introduction

In vertebrates, locomotion and movements are achieved through the generation of muscular forces that are then transmitted to joints. The force transmission (mechanotransduction), which involves the storage, release and dissipation of energy, is provided by connective tissues such as ligaments and tendons (Alexander, 1983; 1984). Thus, in these tissues viscoelasticity is a fundamentally important feature, as both viscous (time-dependent) and elastic (time independent) contributions determine how ligaments and tendons accomplish their function as mechanotransducers. The mechanical behavior of these connective tissues is directly related to their complex hierarchical structure and to their specific macromolecular components (Silver et al., 2001b).

The lowest hierarchical scale of several load-bearing collagenous tissues is represented by collagen type I molecules, which forms the most abundant protein building block in vertebrates, and is the principal protein that provides mechanical stability, elasticity and strength to tendons and ligaments (Kadler et al., 2007; Fratzl, 2008). Collagen molecules are triple helical protein chains with a length of ≈300 nm and a diameter of about 1.5 nm. Collagen molecules are assembled by a parallel staggering into fibrils, which have a thickness in the range from 50 to a few hundred nanometers. Within fibrils, collagen molecules are covalently cross-linked, which is an important aspect for the mechanical standpoint, since cross-links transfer load from one molecule to another. At the next level of the hierarchy, multiple fibrils make up the collagen fiber, formed with the aid of cross-linking macromolecules such as proteoglycans.

At the macro-scale (i.e., whole tissues and collagen fibers), the mechanical investigation of collagen-based tissues has been performed for several decades. As a consequence, it is well known that collagen-rich tissue presents viscoelastic behavior, as established from a larger number of creep and relaxation tests that have been performed on both tendons and ligaments (Rigby et al., 1959; Wang and Ker, 1995; Sasaki et al., 1999). Based on experimental data, and

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starting from the pioneering work of Fung (1967), several constitutive and structural models have been developed to model the viscoelastic behavior of collagen at the tissue scale (Haut and Little, 1972; Egan, 1987; Woo et al., 1993; Puxkandl et al., 2002; Pena et al., 2008; Screen et al., 2011).

On the other hand, fewer studies focused on the viscoelastic properties at smaller scales. At the fibril level, earlier investigations have been performed through small angle X-ray scattering (Sasaki and Odajima, 1996a) to assess the fibril deformation inside tissue specimens. However, in this setup the load is not directly applied to single fibril and hence the response is averaged over several fibrils. Direct experimental studies of single fibrils are relatively recent and made possible due to the use of atomic force microscopy or micromechanical systems which allowed the study of the elastic (Eppell et al., 2006; Van Der Rijt et al., 2006; Shen et al., 2008, 2010; Svensson et al., 2010a) and viscoelastic properties of collagen fibrils (Svensson et al., 2010b; Shen et al., 2011).

Ultimately, at the molecular level several studies have been performed using both experimental and computational procedures; however these investigations focused solely on the elastic properties (Harley et al., 1977; Cusack and Miller, 1979; Hofmann et al., 1984; Sasaki and Odajima, 1996b; Sun et al., 2002; Buehler, 2006; Gautieri et al., 2008, 2009a, 2010) and none has described the single molecule viscoelastic properties. Table 1 summarizes the viscoelastic mechanical properties found for the fibrillar and molecular hierarchical scales of collagenous tissues.

As shown above, despite the abundant investigation of the viscoelastic behavior at the macro-scale and the very recent works on collagen fibril viscoelasticity, relatively little is known about the molecular and fibrillar origin of the time-dependent properties of collagenous tissues. It has been speculated that the elastic behavior of these tissues is due to the stretching of cross-linked collagen molecules and thus fibrils, whereas the energy dissipation (the viscous behavior) is thought to involve sliding of molecules and fibrils by each other during the tissue deformation (Silver et al., 2001a,2001b). However, there is still no clear understanding of the mechanisms behind the viscoelastic behavior of collagenous tissue, and on the role of each hierarchical scale in determining the overall mechanical properties. An important outstanding question is whether or not molecular-level load relaxation effects are important for the viscoelastic properties measured at larger scales.

In order to investigate the molecular origin of collagen viscoelasticity, here we propose an in silico approach to model collagen molecules from the level of amino acids upwards. The aim of this work is to obtain quantitative data on the time-dependent mechanical behavior of single collagen molecules in order to assess molecular-level viscoelastic properties, and to scale up properties from there to larger tissue levels. We consider a segment of the collagen molecule and perform in silico creep tests (i.e., constant load tests), fitting the deformation response over time with a Kelvin–Voigt (KV) model. The KV model is a simple constitutive model consisting of a spring and a dashpot in parallel (see Fig. 1), where this approach allows us to compute the Young's modulus and, for the first time, the viscosity and characteristic relaxation time of a single collagen molecule.

2. Results and discussion

2.1. Viscoelasticity of fully solvated (wet) and dry collagen molecules

In silico creep tests of solvated (wet) and dry single collagen peptides are performed by applying an instantaneous constant force ranging from 300 pN to 3000 pN. The engineering strain of the molecule is monitored over time, showing an exponential increase until asymptotic value is reached (see Fig. 2). This is the behavior typical of simple viscoelastic materials that can be characterized by a Young's modulus E, responsible for the elastic response, and a viscosity η, responsible for the viscous behavior. In order to determine the viscoelastic properties of single collagen molecule, we use the Kelvin–Voigt model to fit the strain-time data. Our results show that collagen molecule presents a non-linear elastic behavior, since the Young's modulus depends on the external load. In particular, for the loading conditions considered here E ranges from 6 to 16 GPa in solvated conditions (Fig. 3a) and from 10 to 19 GPa in the dry environment (Fig. 3b), a finding in good agreement with previous experimental (Harley et al., 1977; Cusack and Miller, 1979; Sasaki and Odajima, 1996b; Sun et al., 2002) and modeling studies (Gautieri et al., 2008, 2009a; Srinivasan et al., 2009).

Here we find that the collagen molecule viscosity has an average value of $3.84. \pm 0.38$ Pa·s in solution (Fig. 3c), whereas a value of 2.64 ± 0.38 Pa·s is found for non-solvated protein (Fig. 3d). We observe that the viscosity is not significantly affected by the external

Table 1
Viscoelastic properties of solvated collagen fibrils and collagen molecules.

Fibril			
	Young's modulus (GPa)	0.43	X-ray diffraction (Sasaki and Odajima, 1996a)
	Average value 0.9 GPa	0.4–0.5	MEMS stretching (Eppell et al., 2006)
		0.2–0.5	AFM testing (Van Der Rijt et al., 2006)
		0.86 ± 0.45	MEMS testing (Shen et al., 2008)
		0.47 ± 0.41	MEMS testing (Shen et al., 2010)
		2.89 ± 0.23	AFM testing (Svensson et al., 2010a)
		1.87–1.94	AFM testing (Svensson et al., 2010b)
		0.3–1.2	Atomistic modeling (Gautieri et al., 2011)
		0.12 ± 0.05	MEMS testing (Shen et al., 2011)
	Viscosity (GPa·s)	0.09–1.63	MEMS testing (Shen et al., 2011)
	Relaxation time (s)	7–102	MEMS testing (Shen et al., 2011)
Molecule			
	Young's modulus (GPa)	≈ 9	Brillouin light scattering (Harley et al., 1977)
	Average value 5.4 GPa	≈ 5.1	Brillouin light scattering (Cusack and Miller, 1979)
		3–5.1	Estimate from persistence length (Hofmann et al., 1984)
		2.9 ± 0.1	X-ray diffraction (Sasaki and Odajima, 1996b)
		0.35–12	Optical trap (Sun et al., 2002)
		≈ 7	Reactive atomistic modeling (Buehler, 2006)
		4.59 ± 0.38	Atomistic modeling (Gautieri et al., 2008)
		≈ 4	Atomistic modeling (Gautieri et al., 2009a)
		4.62 ± 0.41	Coarse-grain modeling (Gautieri et al., 2010)
		6–16	Atomistic creep test [present work]
	Viscosity (GPa·s)	$(3.84. \pm 0.38) \cdot 10^{-9}$	Atomistic creep test [present work]
	Relaxation time (s)	$\approx 0.5 \cdot 10^{-9}$	Atomistic creep test [present work]

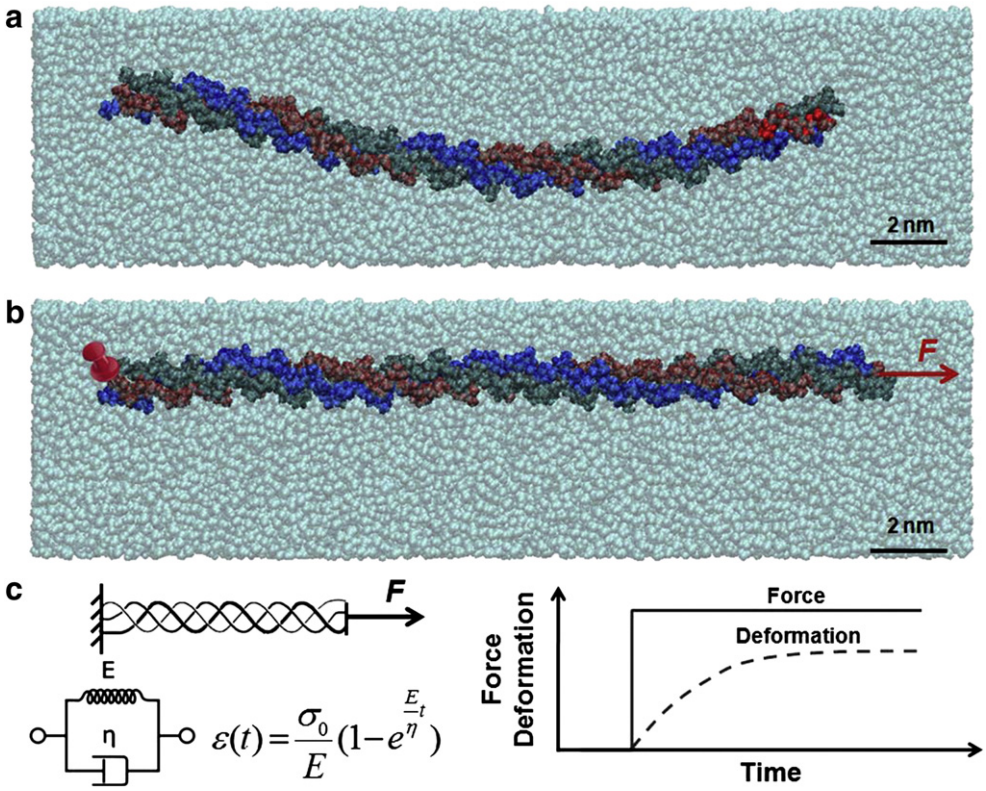

Fig. 1. Snapshots of the collagen peptide in water box. Panel a shows the conformation of the full atomistic model of a $[(GPO)_{21}]_3$ collagen peptide solvated in water box and equilibrated for 30 ns. After equilibration the molecule is subjected to virtual creep tests: one end of the collagen peptide is held fixed, whereas the other end is pulled with constant force (from 300 pN to 3000 pN) until end-to-end distance reaches equilibrium (Panel b). Panel c shows a schematic of the creep test; a constant force is applied instantaneously to the molecule and its response (deformation over time) is monitored. The mechanical response of collagen molecule is modeled using a KV model, from which molecular Young's modulus (E) and viscosity (η) are calculated.

load. Based on the elastic modulus and the viscosity, the characteristic relaxation time (defined, for a KV model, as η/E) is calculated, and we obtain values in the range 0.24–0.64 ns and 0.13–0.27 ns for solvated and dry molecule, respectively. Although the elastic behavior has been previously investigated, no experimental data is available for the viscous component of a single collagen molecule. This could be attributed to the very fast relaxation time (on the order of nanoseconds) of single molecules, which hinders its assessment using available experimental techniques. However, recent work (Nakajima and Nishi, 2006) demonstrated that atomic force microscopy (AFM) can be successfully used to assess the viscoelastic properties of a single polypeptide chain, in this case polystyrene. Within this technique, referred to as "nanofishing" by the authors of that study, an AFM cantilever is mechanically oscillated at its resonant frequency during

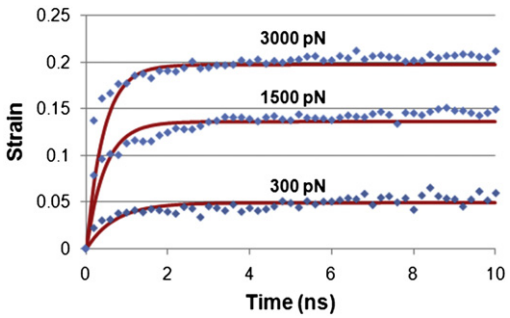

Fig. 2. Single molecule creep test. Mechanical response of solvated collagen molecule to creep tests for three cases with increasing value of external force. Dots represent the experimental data, whereas curves represent the fitted curves using a Kelvin–Voigt model.

the stretching process. This enables the estimation of stiffness and viscosity of a single polymer chain with the use of a phenomenological model (the authors used a KV model). The polystyrene viscosity per unit length reported by the authors is $2.6 \cdot 10{-}9$ kg/s, which agrees well with our findings for collagen molecules, i.e., $\approx 40 \cdot 10{-}9$ kg/s (considering a peptide length of 20 nm). The slightly higher viscosity could be attributed to the fact that the polystyrene chain is a single strand, while the collagen peptide is in a triple helical configuration. With regards to collagen viscosity, it is worth mentioning early birefringence and viscoelastic studies based on solutions of collagen (Ananthanarayanan and Veis, 1972; Bernengo et al., 1983; Nestler et al., 1983). These pioneering studies have been helpful to determine several molecular parameters of collagen, such as molecular radius, translational diffusion coefficient, Young's modulus, persistence length, intrinsic viscosity and flexural relaxation time. Of particular interest for our work are the calculated viscoelastic parameters. These studies found a Young's modulus in the range of $\approx$4 GPa, which well-matches the value found in our work. The intrinsic viscosity, on the other hand, is a measure of a solute contribution to the viscosity of a solution (and measured in ml/g). This parameter, referring to a diluted solution of collagen molecules, has however no clear direct correlation with the viscosity we measure in our work, which is the viscosity of the ideal dashpot modeling the collagen molecule under tensile loading.

The difference between the properties of solvated and dry collagen molecules can be attributed to the differences in the hydrogen bond (H-bond) patterns. We observe that the intramolecular H-bonds do not change as a function of time during the creep tests, nor do they change as a function of the external load (see Fig. 4a). However, the formation of H-bonds is highly influenced by the presence of water around the molecule. Indeed, when in solution, the

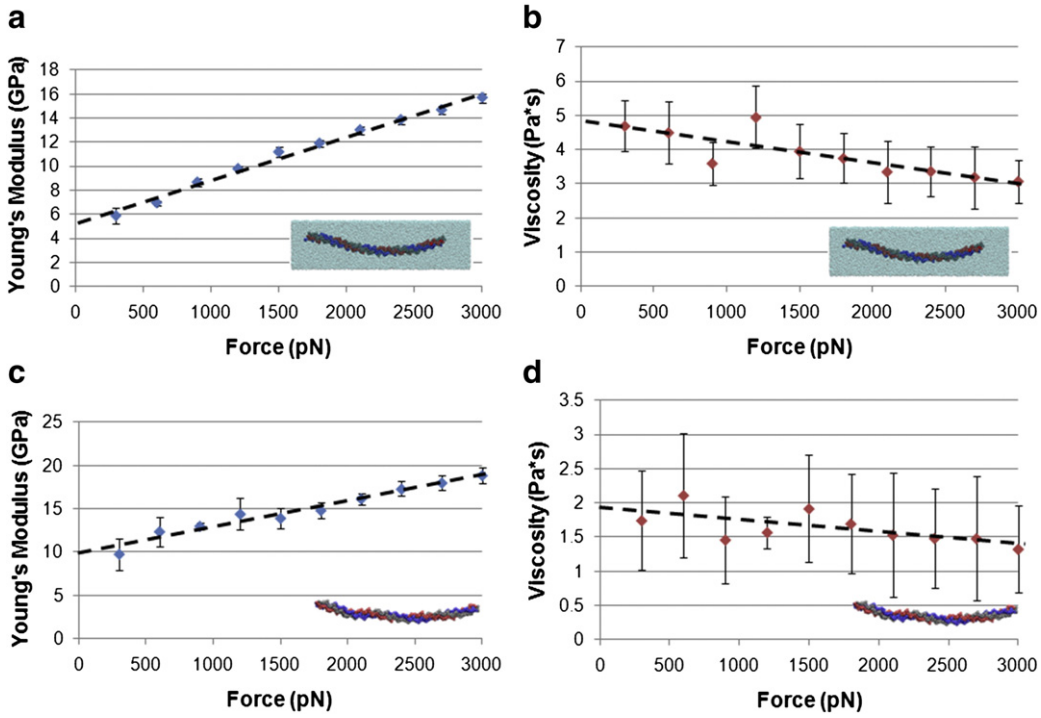

Fig. 3. Young's modulus and viscosity as a function of the applied stress. Creep tests show that the collagen molecule elastic component is non-linear, since the molecular Young's modulus increases with increasing load, ranging from 6 GPa to 16 GPa for the solvated system (Panel a) and from 10 to 19 GPa in dry environment (Panel b). The calculated viscosity is approximately constant through the applied loading range, with an average value of 3.84 ± 0.38 Pa·s for the solvated molecule (Panel c) and a value of 2.64 ± 0.38 Pa·s for dry collagen molecule (Panel d).

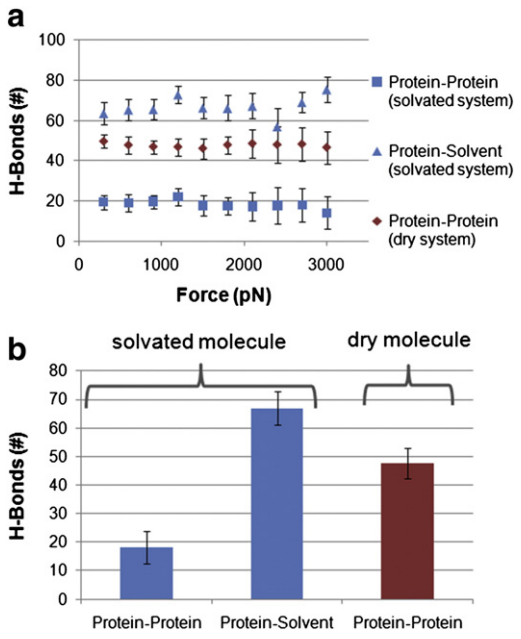

Fig. 4. Hydrogen bond analysis during relaxation. Number of intramolecular and protein-solvent H-bonds as a function of the external force, calculated averaging data from three independent SMD simulations, showing that the number of H-bonds is not affected by the external load (panel a). When in solution, collagen molecules form less intramolecular H-bonds (18 ± 5) than dry molecules (47 ± 5); however, solvated collagen molecules form a large number of protein-solvent H-bonds (67 ± 6), which are absent in the dry environment (Panel b). The larger number of intramolecular H-bonds could thus explain the higher Young's modulus found for collagen molecules in dry environment. For identification of H-bonds, an angle donor-hydrogen-acceptor of 30° and a cutoff distance of 35 Å between the donor and the acceptor are considered in a geometric analysis.

collagen molecule forms less intramolecular H-bonds than the dry molecule, whereas it forms several protein-solvent H-bonds which are absent in the dry environment (see Fig. 4b). Thus, the higher Young's modulus found for the dry molecule can be attributed to the larger number of intramolecular H-bonds, which increases the backbone rigidity. On the other hand, the higher viscosity of solvated vs. dry molecule can be explained considering the H-bonds that the solvated molecule forms with the surrounding water layers. The breaking and reformation of these protein-solvent H-bonds during the creep tests would result in a drag force on the stretching collagen molecule, causing the observed increased viscosity.

The work reported above is based on a peptide featuring only Gly-Pro-Hyp triplets, thus representing an archetypical collagen model, since this triplet is the most common and characteristic for collagen. Similar peptides (also called host-guest peptides) have been widely used to experimentally study properties of collagen, as reviewed in Brodsky et al. (2008). However, it is interesting to investigate the effect of sequence on the viscoelastic parameters. As a preliminary test to assess the effect of sequence, other than Gly-Pro-Hyp, on the viscoelastic properties of collagen molecule, we consider a peptide with real sequence, namely helical region comprised between residue 724 and 786. The sequence of this peptide features some Gly-Pro-Hyp triplets along with a more varied sequence, including charged amino acids. The creep tests show that this peptide has a viscoelastic behavior similar to the Gly-Pro-Hyp peptide (data not shown). The Young's modulus is found to be slightly lower with respect to the reference peptide, for all force levels. This could be due to the fact that the Gly-Pro-Hyp sequence is generally the most stabilizing for the triple helix configuration and thus would lead to a stiffer behavior. Likewise, the viscosity shows some differences with regards to the reference peptide, which may be due to the specific sequence considered. However, the values obtained for the viscoelastic parameters do not differ significantly in comparison with the values found for the archetypical collagen. Thus, even if the Gly-Pro-Hyp triplet is not the only triplet found in collagen, it seems

representative enough to allow us to draw general conclusions on the viscoelastic properties of collagen at the molecular level. Future work could look further and investigate the issue of sequence dependence of viscoelastic properties.

We use the KV model to fit the extension-time curves since this model is the most basic viscoelastic mechanical model available and it provides an excellent fit to the measured mechanical response. It is of great interest to discuss whether the two elements of the KV model, i.e. the purely elastic spring and the purely viscous dashpot, have an actual physical meaning. A likely explanation would be that the elastic spring corresponds to the protein backbone, while the damping effect could be attributed to the interchain H-bonds. The backbone deformation include dihedral, angle and bond deformation, which are terms expressed by harmonic (or similar) functions in the molecular dynamics force field, and thus result in an elastic response to stretching. On the other hand, the viscous behavior may be due to the breaking and reforming of H-bonds, in particular H-bonds between the three collagen chains. In earlier works, statistical mechanics based theories, and in particular Bell's model (Bell, 1980), has been applied to study the breaking of H-bonds (see, e.g. reference Buehler and Ackbarow, 2007 and Fig. 5). It has been shown that the velocity at which an H-bond breaks is a function of the external force:

$$v = \frac{x_b}{\tau_0} e^{-\left[\frac{E_b - F \cdot x_b \cdot \cos\theta}{k_b T}\right]} \tag{1}$$

where v is the velocity at which the H-bond breaks, x_b is the distance between the equilibrium state and the transition state, τ_0 is the reciprocal of the bond natural frequency, E_b is the bond energy, F is the applied force, θ is the angle between the direction of the reaction pathway of bond breaking and the direction of applied load F, k_b is the Boltzmann constant and T is the temperature. Eq. (1) shows that the mechanical response of the H-bonds is time-dependent, matching the behavior of the dashpot in the KV model very well (for which the stress is a function of the loading rate). Therefore the time-dependent rupture of H-bonds are likely responsible for the dissipative behavior observed in collagen molecule mechanics.

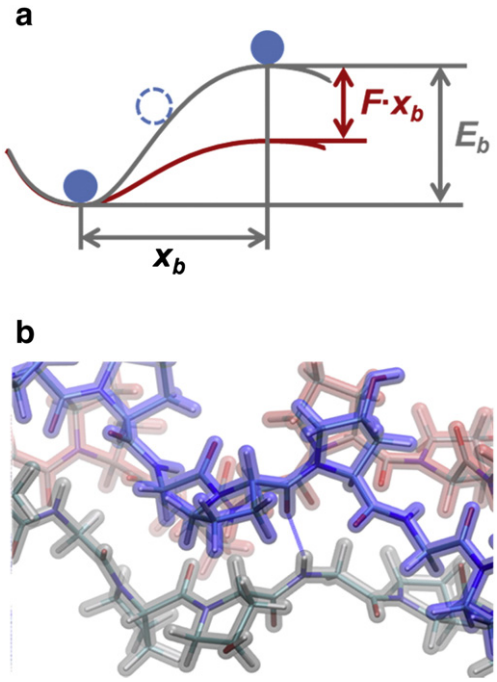

Fig. 5. Statistical theory to predict bond rupture mechanics. The graph depicts the energy as a function of a deformation variable (a), along a particular pathway that leads to bond rupture, where E_b is the energy barrier corresponding to the transition state. Panel b shows an interchain H-bond in collagen.

2.2. Comparison of molecular and fibrillar viscoelasticity

The systematic comparison of collagen molecule viscoelastic properties with those of the collagen fibrils (see Table 1) yields two main observations:

1. The average Young's modulus decreases about six-fold from the single molecule level (5.4 GPa) to the fibrillar scale (0.9 GPa).
2. The viscosity of collagen molecules (3.84 Pa·s) is several orders of magnitude lower than the viscosity of fibrils (0.09–1.63 GPa·s). As a result, the characteristic relaxation time of the molecule ($\approx$0.5 ns, given by η/E) is several orders of magnitude lower that the value found for the fibril (7–102 s).

In order to explain these observations, we attempt to upscale from a single molecule to fibril considering that collagen fibrils are assemblies of collagen molecules connected by covalent cross-links (see Fig. 6a) and arranged in a quasi-hexagonal array in cross-section (Orgel et al., 2006) (Fig. 6b). Thus, the fibril can be modeled, in a first approximation, as a collection of KV elements arranged both in series and in parallel (Fig. 6c), where each of these elements account for a single collagen molecule.

We model the collagen molecule as a KV material, one of the simplest models for viscoelastic materials, which can be represented by an ideal viscous damper and an ideal elastic spring connected in parallel (see Fig. 1c). The governing equation of the KV model is:

$$\sigma(t) = E\varepsilon(t) + \eta\dot{\varepsilon}(t) \tag{2}$$

where $\sigma(t)$ is the stress as a function of time t, $\varepsilon(t)$ is the engineering strain, E is the material Young's modulus and η is the material viscosity. Considering an instantaneous constant stress σ_0 applied to the KV model and solving the differential equation for the strain we obtain:

$$\varepsilon(t) = \frac{\sigma_0}{E}\left(1 - e^{-\frac{E}{\eta}t}\right) \tag{3}$$

where the inverse of E/η represents the characteristic time of the material. The KV model behavior (Eq. (2) and Eq. (3)) can be expressed in terms of force and deformation, instead of stress and strain:

$$\frac{F}{A} = \frac{kl}{A}\frac{\Delta l(t)}{l} + \eta\frac{\Delta\dot{l}(t)}{l} \tag{4}$$

$$\Delta l(t) = \frac{F}{k}\left(1 - e^{-\frac{k/A}{\eta/l}t}\right) \tag{5}$$

where F is the applied constant force, $\Delta l(t)$ is the deformation as a function of time t of the KV element of area A and length l, k is the spring elastic constant and η the viscosity of the damper. When N such elements are connected in series, the total deformation experienced by the system is the sum of the individual deformations:

$$\Delta l(t) = \Delta l_1(t) + \ldots + \Delta l_N = \frac{F}{k_1}\left(1 - e^{-\frac{\frac{k_1}{A_1}}{\frac{\eta_1}{l_1}}t}\right) + \ldots + \frac{F}{k_N}\left(1 - e^{-\frac{\frac{k_N}{A_N}}{\frac{\eta_N}{l_N}}t}\right). \tag{6}$$

Assuming that the N elements are identical (with the same spring constant k, viscosity η, area A and length l), the behavior of the system results:

$$\Delta l(t) = \frac{NF}{k}\left(1 - e^{-\frac{k/A}{\eta/l}t}\right). \tag{7}$$

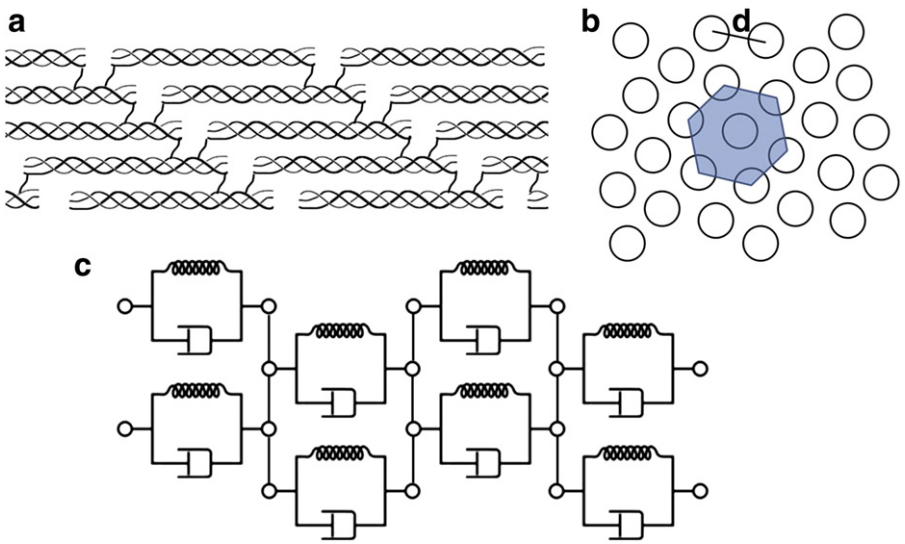

Fig. 6. Schematic of collagen fibril and viscoelastic model based on KV elements. Collagen type I form supramolecular structures, the collagen fibrils, where molecules are assembled in a staggered fashion by about 67 nm, i.e. the so-called D-period (Panel a). Laterally, the triple-helices are arranged in a quasi-hexagonal array in cross-section leading to fibril with diameters in the range 30–300 nm (Panel b). The molecular lateral distance d depends on the hydration states: the typical lateral spacing between molecules in the fibrils decreases from about 1.6 (hydrated state) to 1.1 nm (dry state) (Fratzl et al., 1993). Within the fibril, collagen molecules are connected by covalent cross-links that ensure load transmission throughout the fibril structure. As a first approximation, the collagen fibril can be assumed as an assembly of multiple KV elements (each one representing a collagen molecule), connected both in series and in parallel (Panel c).

Thus, when N identical KV elements are connected in series, the system itself behaves as a KV material with overall spring constant $k_{sys}=k/N$, whereas the characteristic time of the system remains the same as that of the unit elements. On the other hand, when N KV elements act in parallel, the total strain experienced by the system is the sum of the strain of each element, which can be expressed as:

$$\frac{F}{A}=\frac{F_1}{A_1}+\ldots+\frac{F_N}{A_N}=\frac{k_1 l_1}{A_1}\frac{\Delta l(t)}{l_1}+\eta_1\frac{\Delta\dot{l}(t)}{l_1}+\ldots+\frac{k_N l_N}{A_N}\frac{\Delta l(t)}{l_N}+\eta_N\frac{\Delta\dot{l}\,(t)}{l_N}. \tag{8}$$

Assuming that the N elements are identical (with the same spring constant k, viscosity η, area A and length l), the behavior of the system results:

$$\Delta l(t)=\frac{F}{Nk}\left(1-e^{-\frac{k/A}{\eta/l}t}\right). \tag{9}$$

Thus, when N identical KV elements are connected in parallel, the system behaves as a KV material with overall spring constant $k_{sys}=k\cdot N$. Also in the parallel case, the characteristic time of the system is the same as that of the unit elements.

Within the fibril, collagen molecules are assembled in series staggered by a fixed period of about 67 nm (the so-called D-period). Laterally, the triple-helices are arranged in a quasi-hexagonal array in cross-section, with a lateral distance d (see Fig. 6b). Thus, in a fibril of cross-sectional area A_f, the number N_p of parallel molecules of area A_m results:

$$N_p=\frac{A_f\rho}{\pi(d/2)^2} \tag{10}$$

where ρ is the density of the hexagonal arrangement ($\rho\approx 0.9096$), i.e. the fraction of the plane occupied by identical circles hexagonally arranged. On the other hand, in a fibril of length L_f, the number N_s of serial molecules staggered by a distance s ($\approx$67 nm) results:

$$N_s=\frac{L_f}{s}. \tag{11}$$

The fibril elastic constant k_f, considering N_p molecules acting in parallel and N_s molecules acting in series, results:

$$k_f=k_s\frac{N_p}{N_s}=\frac{E_m A_m}{s}\frac{A_f\rho}{\pi(d/2)^2}\frac{s}{L_f} \tag{12}$$

where k_s is the elastic constant of the resistant element of length s, and E_m it's the molecular Young's modulus. Finally, the Young's modulus of the fibrils results:

$$E_f=k_f\frac{L_f}{A_f}=\frac{E_m A_m A_f\rho s}{s\pi(d/2)^2 L_f}\frac{L_f}{A_f}=\frac{E_m A_m\rho}{\pi(d/2)^2}. \tag{13}$$

We can assume $E_m=6$ GPa (10 GPa for dry molecules), $A_m=9.5\cdot10^{-19}$ m^2 (given by a molecular radius of 0.55 nm) and $\rho=0.9096$. Concerning the value of the intermolecular lateral distance d, Fratzl et al. (1993) found a lateral spacing of 1.6 nm for wet collagen and 1.1 nm for dry collagen. From Eq. (13) we obtain a Young's modulus of 2.5 GPa for wet collagen fibrils, a value that is in very good agreement with experimental and computational results (in the range 0.12–2.8 GPa, see Table 1). In the case of dry collagen the fibril Young's modulus results 9.0 GPa, thus a 3–4 fold increase with respect to wet collagen. A similar result has been found in previous works where the authors reported a 2–3 (Svensson et al., 2010a), a 4–10 (Van Der Rijt et al., 2006) and a 2–7 (Gautieri et al., 2011) fold increase for the fibril Young's modulus upon dehydration. Thus, it can be concluded that the elastic component of the fibril mechanical behavior is, in first approximation, well estimated assuming the fibril to be formed by a collection of KV elements (each representing a single collagen molecule) arranged in series (due to the D-periodic staggering, see Fig. 6a) and in parallel (due to the quasi-hexagonal packing, see Fig. 6b).

On the other hand, according to the model outlined above the characteristic time of such collection of KV elements is expected to be the same as that of the single KV element. In our case, however, we observe that the characteristic time of a collagen fibril (7–102 s) is several orders of magnitude higher than the value found here for the single collagen molecule ($\approx$0.5 ns). This implies that the viscous properties of collagen fibrils are not directly dictated by the single molecule behavior, but are largely dependent on other mechanisms,

most likely the water-mediated shearing of the molecules within the fibril. This finding supports previous hypotheses based on mechanical tests on self-assembled collagen fibers (Silver et al., 2001a). In their work, the authors demonstrated a transition of collagen fiber mechanical properties from viscous to elastic depending on the cross-linking content, concluding that at low level of cross-linking the mechanical response is dominated by viscous sliding of collagen molecules and fibrils by each other, while at higher levels of cross-linking the mechanical response is mostly dictated by the elastic stretching of molecules. Our work, for the first time, provide quantitative data to support these hypotheses, showing that the elastic response of fibrils can be largely ascribed to the stretching of cross-linked single molecules, whereas the molecular-level relaxation effects does not determine the viscoelastic properties measured at the fibril level.

The higher hierarchical organization in tissues like tendon is formed by collagen fibers, in which individual collagen fibrils are linked together by proteoglycans (see Fig. 7a). The molecular mechanisms governing the mechanical response at this level are still unclear, although it has been shown (Puxkandl et al., 2002) that the viscoelastic properties of fibers arise due to the stretching of mostly elastic collagen fibrils connected by a mostly viscous matrix (made of proteoglycans). A simple micromechanical model has been proposed in earlier works (Redaelli et al., 2003) to explain the role of proteoglycans as load transfer elements between fibrils. Although this simple model assumes proteoglycans to be covalently linked to collagen and does not consider viscous effects, it is effective in capturing the elastic behavior of collagen fibers. Future works could be aimed at using molecular dynamics approach to assess the interaction between collagen and decorin and rate effects in order to extend the aforementioned model.

Finally, it is interesting to explore whether or not the hierarchical structure of collagen can be regarded as a fractal structure. Some data suggests that this is case from both a structural (see Fig. 7b) and a mechanical point of view. Indeed, within the fibril the viscoelastic response depends on collagen molecules that are stretched during loading and that give the elastic response, whereas the water matrix, though weak non-covalent H-bonds, is likely to contribute to the viscous behavior. At the higher fiber scale, collagen fibrils are the structural features that provide elastic behavior, whereas the proteoglycan matrix provides viscosity by connecting the elastic elements through weak links (in this case, electrostatic and van der Waals interactions between proteoglycans and collagen). Future work could explore these issues further.

3. Conclusion

In this work, we performed in silico creep tests on collagen peptides to investigate the time-dependent mechanical properties of individual collagen molecules. From stress-time curves we observe that collagen molecule behavior can be captured by a simple KV model (consisting in a spring and a dashpot in parallel), thus allowing us to determine single molecule viscoelastic properties. We find a nonlinear elastic component, with a Young's modulus ranging from 6 GPa to 16 GPa, in agreement with previous computational and experimental studies. The time-dependent properties, measured

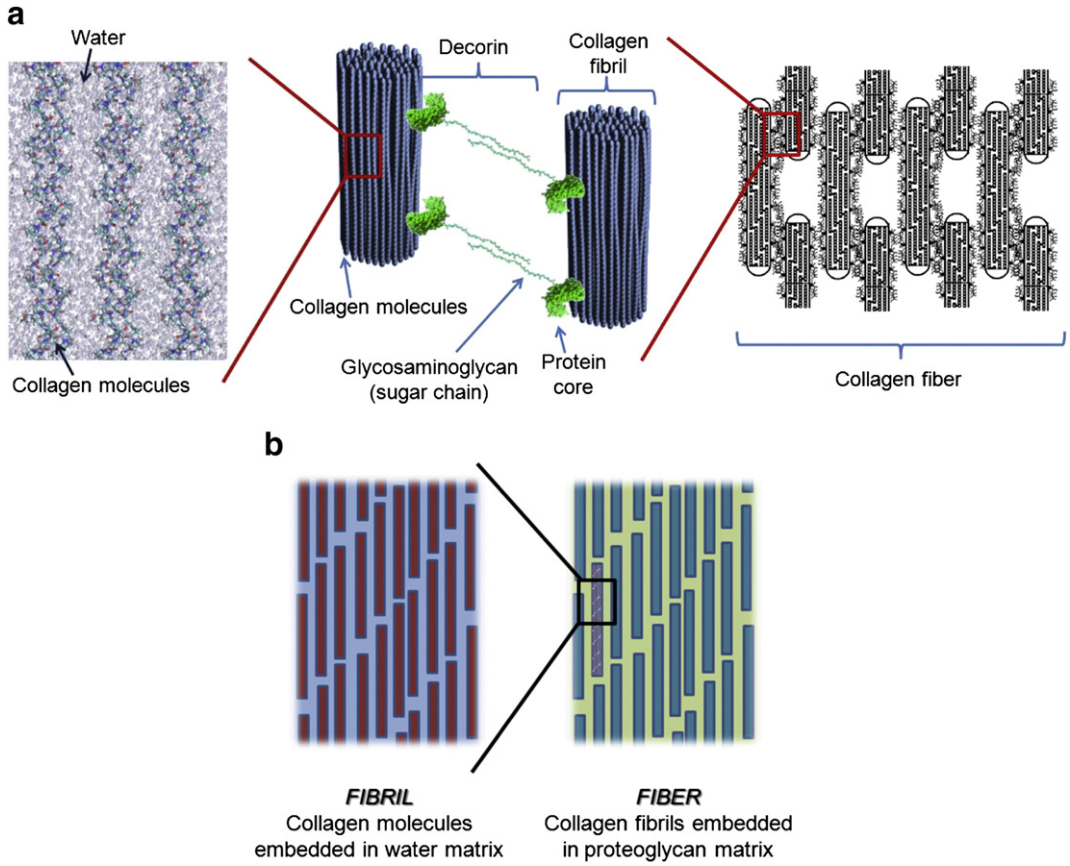

Fig. 7. Schematic of collagen fiber. In tissues like tendons and ligaments collagen fibrils form higher-scale structures, the collagen fibers. Individual fibrils are connected through proteoglycans such as decorin, which are formed by a protein core and a sugar chain and which work as load transfer elements within the fiber. Proteoglycans form an entangled matrix between the fibrils and the protein core is weakly (not covalently) bonded to collagen fibrils, therefore it can detach and reconnect (Panel a). For these reasons it has been suggested (Puxkandl et al., 2002) that the proteoglycan matrix could be the major responsible for the viscous behavior of collagen fibers. Panel b shows a schematic of the fractal structure of collagen tissue, from molecule to fiber.

here for the first time at the single molecule level, show that collagen molecule has a viscosity of ≈4 Pa·s and a relaxation time of ≈0.5 ns. We note that our analysis is based on a model peptide with only glycine-proline-hydroxyproline triplets and thus we expect that natural occurring collagen, which features a more varied primary sequence, might exhibit viscoelastic properties that partially deviate from those shown here.

From single molecule we upscale to the fibril and fiber level, starting from the assumption that the fibril is formed by cross-linked collagen molecules and thus modeling the fibril as a collection of KV elements (each one accounting for one molecule) arranged in series an in parallel. Although this model is quite simplified, it allows us to give a good estimate of the elastic properties of the fibril starting from the Young's modulus of the single molecule. This suggests that the elastic component of fibril mechanical behavior is largely dictated by the stretching of triple helical molecules. On the other hand, this model fails to capture the time-dependence of fibril mechanical response, suggesting that the viscous component does not primarily depend on single molecule relaxation but largely relies on other mechanisms, such as water-mediated sliding of adjacent molecules.

The insight reported in this article is important for our understanding of the role of collagen as a key constituent in the extracellular matrix. In particular, the scale-dependent mechanical stiffness of collagenous tissues, with a systematic drop in the modulus as larger scales of tissues are reached, could be important for tissue development. Future work could, in addition to studies of identifying the origin of viscoelastic effects at larger (fibril and fiber) scales, also focus on the importance of the specific relaxation times identified here for single collagen fibrils. This may include a detailed nanomechanical analysis of how proteins such as integrins bind to collagen, how such molecular adhesions rupture, and may reveal whether or not these time-scales are relevant for the development of tissue, or even for processes such as remodeling. Future work should also address the limitations of time-scales in our molecular dynamics simulations, which are inherently limited to an order of magnitude of less than hundred nanoseconds. Hence, relaxation mechanisms at significantly larger time-scales may not be visible in such simulations. While there exist several new methods to enhance the ability of molecular modeling to sample longer time-scales (e.g. Replica Exchange, Metadynamics, etc.), the extraction of dynamical properties from such methods is difficult. Dynamical information, however, is needed to extract viscoelastic properties of the material.

4. Computational procedures

4.1. Collagen model generation

The viscoelastic mechanical properties of the collagen molecule are investigated using constant force Steered Molecular Dynamics (SMD) simulations, submitting the collagen molecule to pulling along its principal axis with increasing external load. We use the THeBuScr (*T*riple-*He*lical collagen *Bu*ilding *Scr*ipt) code (Rainey and Goh, 2002, 2004) to build a model of the collagen molecule, as done in earlier studies (Gautieri et al., 2009b, 2010; Srinivasan et al., 2010). We choose the simplest model of collagen, with only glycine-proline-hydroxyproline (GPO) triplets on each of the three chains. The collagen model we use, $[(GPO)_{21}]_3$, is truncated to 63 amino acids per chain due to computational limitations, since the full length collagen molecule (300 nm long) is too large for atomistic simulations. This leads to a collagen-like segment with a length of approximately 20 nm. Peptides of comparable length have been used both in earlier computational and experimental studies (Beck et al., 2000; Persikov et al., 2000a,2000b; Gautieri et al., 2008, 2009b; Veld and Stevens, 2008; Srinivasan et al., 2009).

4.2. Collagen model equilibration

Molecular dynamics simulations are performed using the NAMD code (Nelson et al., 1996; Phillips et al., 2005) and the CHARMM force field (MacKerell et al., 1998), which also includes parameters for the hydroxyproline residue. Since the collagen triple helix considered here is truncated, the N-terminals are capped with ACE residues (acetylated N-termini), whereas C-terminals are capped with CT3 residues (amidated C-termini).We investigate the viscoelastic behavior of both dried and solvated collagen molecule. In the latter case the protein is solvated in a 24 nm×6 nm×6 nm water box using TIP3P water molecules for solvent, and ions are included to achieve a ionic concentration of 0.5 mol/L (see Fig. 1a). The total number of atoms of the solvated system is approximately 90,000 atoms. Rigid bonds are applied to constrain covalent bond lengths, thus allowing a time step of 2 fs. Van der Waals interactions are computed using a cutoff for neighbor list at 1.35 nm, with a switching function between 1.0 and 1.2 nm. For electrostatic a similar switching function is used for the dry case, whereas the Particle-Mesh Ewald sums (PME) method is applied to describe electrostatic interactions in the solvated systems. The preliminary energy minimization is performed by using a steepest descent algorithm until convergence. The systems are then equilibrated at a temperature of 310 K (37 °C) and (for the solvated system) at a pressure of 1 atm for 30 ns of molecular dynamics. We observe that the root mean square deviation (RMSD) of the protein backbone reaches a stable value within 15 ns simulation time, thus we assume that the collagen peptide is equilibrated properly at the end of the 30 ns molecular dynamics run. Three different configurations of the protein in the stable RMSD regime (at 20 ns, 25 ns and 30 ns) are extracted as starting points for the subsequent mechanical tests to determine how different equilibration lengths influence the results.

4.3. In silico creep tests

In order to perform constant force SMD simulations, the three N-terminal C_α atoms are kept fixed whereas the three C-terminal C_α atoms are subject to an instantaneous constant force (see Fig. 1b). The total force applied to the C-terminal ranges from 300 pN to 3000 pN. This setup mimics a single molecule creep test, in which an instantaneous load is applied and the strain response in time is observed. The creep test simulations are run for 10 ns, a time which is found enough to reach asymptotic deformation. As described in the text we model the collagen molecule as a KV material. Strain-time curves obtained for collagen molecules during SMD simulations are fitted with Eq. (3) in order to estimate E and η. The instantaneous stress σ_0 is calculated dividing the applied force by the cross-sectional area of collagen, assuming a round cross-section and a radius of 0.55 nm. The value of the radius is determined from dry collagenous tissue in which the lateral distance between molecules is found to be 1.1 nm (Fratzl et al., 1993). We choose to characterize the collagen molecule with a KV model since it is straightforward to implement creep tests using the SMD approach. Another choice would have been to use the Maxwell model (represented by an ideal viscous damper and an ideal elastic spring connected in series). In order to determine the Young's modulus and viscosity with such model we would need to perform stress relaxation tests, in which an instantaneous strain is applied and the stress over time is monitored. However, while creep tests are possible through constant force simulations (which can be directly done using the NAMD code), an equivalent tool to monitor the stress relaxation after imposing a constant strain is not possible, limiting our ability to perform stress relaxation tests.

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