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Elasticity and Inverse Temperature Transition in Elastin

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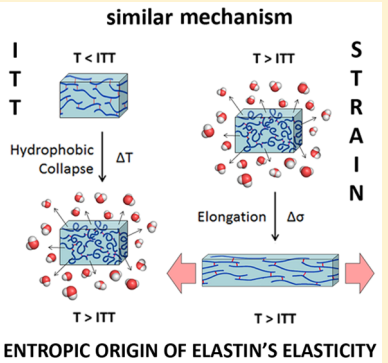
Supporting Information

ABSTRACT: Elastin is a structural protein and biomaterial that provides elasticity and resilience to a range of tissues. This work provides insights into the elastic properties of elastin and its peculiar inverse temperature transition (ITT). These features are dependent on hydration of elastin and are driven by a similar mechanism of hydrophobic collapse to an entropically favorable state. Using neutron scattering, we quantify the changes in the geometry of molecular motions above and below the transition temperature, showing a reduction in the displacement of water-induced motions upon hydrophobic collapse at the ITT. We also measured the collective vibrations of elastin gels as a function of elongation, revealing no changes in the spectral features associated with local rigidity and secondary structure, in agreement with the entropic origin of elasticity.



Elastin is a mammalian extracellular matrix protein, which plays an essential role in providing elasticity and resilience to a wide variety of tissues including: arteries, lungs, skin, elastic cartilage, elastic ligaments, and the bladder.[1] Elastin is a nearly ideal elastomer up to an extension of ∼70%,[2] with a Young's Modulus of 300−600 kPa and a maximum strain before failure up to 100% − 220%.[3] It is also one of the most stable proteins known[4] with an *in vivo* half-life of 70 years and able to undergo billions of cycles of extension and recoil without mechanical failure.[5] These properties are important, as the production of elastin in the body drops quickly after the early years of life, meaning that damaged elastin cannot be easily replaced. Loss of elastin's mechanical properties through mutation or other dysfunction can lead to a number of disease states such as emphysema, cutis laxa, and supravalvular aortic stenosis.[6] Because of this, elastin and elastin derived peptides show promise as a biomaterial in a wide range of applications.[7−15] However, even with its wide use in therapeutic applications, fundamental questions as to the biophysical origins of its advantageous mechanical properties remain.

According to the classical model for rubbery materials,[16] the polymer (polypeptide) chains adopt randomly coiled configurations which are chemically linked at various points. Upon deformation, the restoring force of the material arises mainly from the decrease in entropy associated with the stretching of the chains from random and relatively compact configurations to more extended structures, which are more aligned in the direction of strain.[8−10] However, other studies claim to find large enthalpic variations associated with the deformation of elastin when swollen and in contact with excess water.[14,15] Observations on the macroscopic scale point to a critical role of water. Elastin becomes hard, brittle, and glassy in the absence of water,[17] a fact that has been used to argue that its elastomeric properties are closely related to the effects of its hydrophobic hydration.

Another intriguing feature of elastin is the inverse temperature transition (ITT) which occurs at ∼295 K, at which point the low temperature extended form of the molecule[18] contracts into a compact form.[19−21] Above its transition temperature, elastin can be described as a "dynamic-compact amorphous globule" with fluctuating turns, distorted β-strands, buried hydrophobic residues, and main-chain polar atoms involved in hydrogen bonding with water.[18] At the ITT hydrophobic

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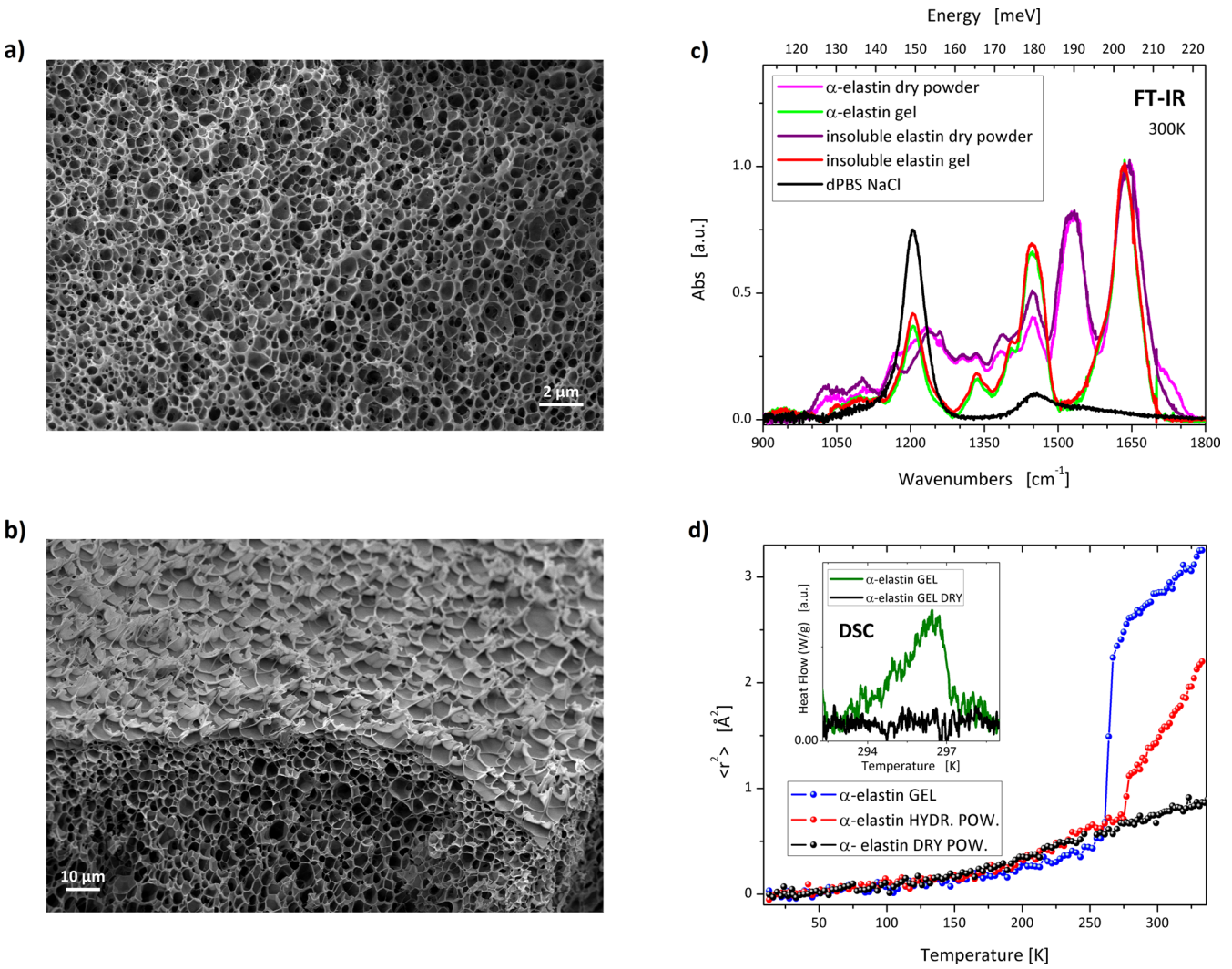

Figure 1. Physical characterization of elastin samples. SEM images obtained for α- elastin (a) and insoluble elastin (b) gels show the expected porous structure. Magnification used: (a) 11.60k; (b) 1.79k. (c) FT-IR spectra of α- and insoluble elastin in the form of dry powders and cross-linked hydrogels at 300 K. Spectra were normalized to the maximum height of the Amide I band. (d) Temperature variation of mean-squared displacements $\langle r^2 \rangle$ for α-elastin in the form of dry powder (black symbols), hydrated powder (red symbols), and cross-linked hydrogel (blue symbols). See Figure S2 for raw elastic intensities and a zoom-in of the low temperature region of the MSD shown in panel d. Inset: conformational transition of fully hydrated α-elastin hydrogel (green line) shown by calorimetry data. The lyophilized gel (black line) does not present any transition in this temperature range.

collapse and simultaneous expulsion of water molecules associated with the nonpolar side-chains of the protein are results of a reversal in the balance between the entropy gained upon disordering and the enthalpic cost on removal of stabilizing interactions. This is connected to the uncommon amino acid composition of elastin, with about 75% hydrophobic residues (Gly, Val, Ala), as well as its extensive cross-linking, which make elastin relatively insoluble in water.[22] Moreover, it has even been suggested that a common mechanism is shared for both the ITT and elasticity of elastin.[23]

Investigations of the biophysical properties of elastin have proven to be challenging, however. Even the structure of elastin remains elusive due to the highly amorphous and cross-linked nature of the protein and the high mobility of its backbone (on a subnanosecond time scale).[24] In addition, numerous experiments conducted on soluble forms of elastin, using circular dichroism, FT-Raman, and electron microscopy[25−30] have led to a variety of different interpretations and no consensus on the secondary structure of elastin. Contrasting interpretations of the data indicate a variety of different motifs, such as polyproline II, type II β-turns, β-sheet, unordered, or α-helical motif, as preferred conformations of elastin and elastin-like peptides (ELPs).[18,23,25−30] In spite of the different explanations, experimental and molecular dynamics (MD) simulations results seem to suggest that any secondary structure is short and local, such as turns and distorted β-structures, consistently with elastin's ability to reversibly deform. Dynamical approaches offer an alternative methodology to interrogate the origins of these properties; unfortunately, there has been relatively little research to date into the dynamics of elastin materials.[23,31]

In this work, we explore both the ITT and the physical origin of elastin's superb mechanical properties. Our investigation is based on a dynamical approach utilizing neutron scattering. This technique enables us to isolate the contribution of elastin from that of water due to the remarkable difference (~40 times) in the incoherent scattering cross section between the isotopes of hydrogen. By combining two different neutron

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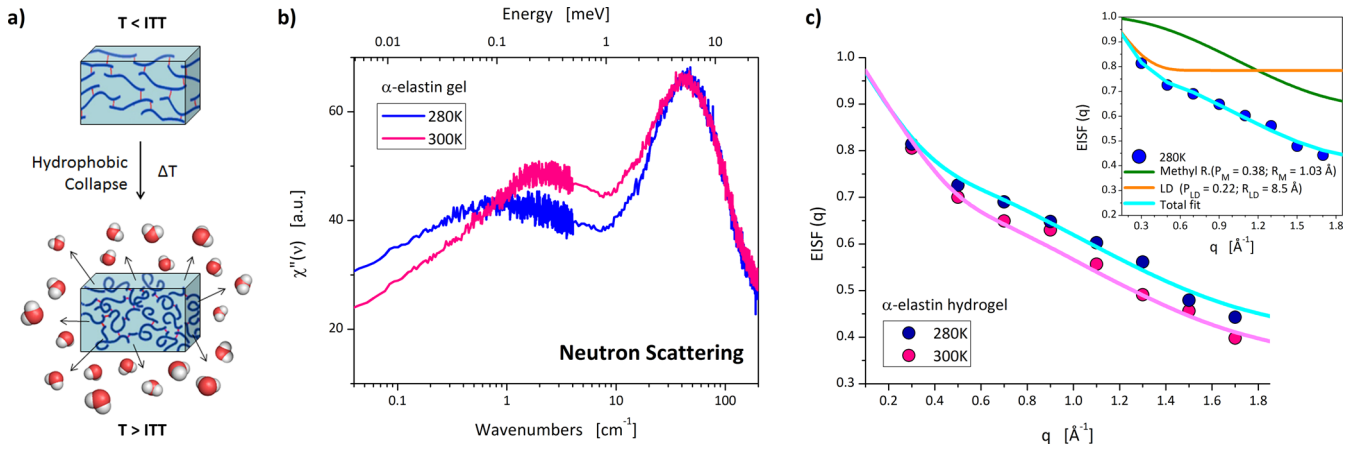

Figure 2. Nanosecond−subpicosecond dynamics of elastin below and above the ITT. (a) Scheme of the ITT of elastin: hydrophobic collapse and expulsion of water molecules. (b) Susceptibility spectra (combined BASIS and CNCS neutron data) for α-elastin hydrogel at 280 K (blue line) and 300 K (pink line). The spectra correspond to q = 1.1 Å^{-1}. (c) EISF(q) for α-elastin hydrogel measured on the BASIS spectrometer (~300 ps) at 280 K (pink circles) and 300 K (blue circles). Symbols are experimental data and lines are the total fits to the data. Inset: EISF(q) of α-elastin hydrogel at 280 K. The green line represents the methyl groups' contribution, the orange line shows the fit with diffusion on a sphere model, and the red blue line (total fit) represents the sum of these two components.

spectrometers, we were able to explore a wide range of dynamics, from hundreds of picoseconds to femtoseconds. This has allowed us to characterize the geometry and amplitude of water-enhanced localized diffusion motions below and above the ITT, as well as looking at low frequency collective vibrations of elastin gels as a function of elongation. These experiments have shown that hydrophobic hydration plays a critical role in both the ITT and the mechanical properties of elastin, and is consistent with the notion that the entropic considerations contributing to the ITT also act as the restoring force for elastin at physiological strains below 70%.

Our studies have made use of α-elastin and insoluble elastin, in the form of dry powders, hydrated powders (h = 0.4, where h = mass of D_2O/mass of protein) and cross-linked hydrogels (h = 1). α-Elastin gels were also analyzed after lyophilization to evaluate effects of the complete removal of water (α-elastin gel dry). Samples were prepared using hydrogenated elastin and D_2O, in order to minimize the solvent contribution to the neutron spectra, enabling us to probe only the dynamics of the protein (D_2O contribution was less than 15% of the total scattering, Table S1). Full details of sample preparation and experimental conditions are reported in the Materials and Methods section in Supporting Information. Cross-linked elastin hydrogels were prepared following the "single-step alkaline protocol" described by Leach et al.[32] The resulting gels are reported to have elastic moduli of ~38 kPa. We have characterized these gels at multiple length scales, using infrared spectroscopy and scanning electron microscopy (SEM) (Figure 1). SEM images of dry α- and insoluble elastin powders showed disordered flakey structures (Figure S1 in Supporting Information). The hydrogel materials (Figures 1a,b) have an interconnected porous structure that has found favorable application for homogeneous cell distribution, penetration, and growth within the 3D scaffold.[33] Analysis of the infrared spectra shows significant overlap between α-elastin and insoluble elastin for dry powders and hydrated hydrogels at 300 K (Figure 1c), suggesting that the two types of the protein adopt very similar conformations.

Temperature- and q-dependent elastic neutron scattering can be used to highlight dynamic transitions in elastin. This was performed using samples of α-elastin as a dry powder, a hydrated powder (h = 0.4), and a hydrated gel (h = 1.0) at temperatures between 10 K and 347 K. This experiment is sensitive to the motion of hydrogen atoms occurring faster than ~300 ps (the resolution of the BASIS spectrometer). The raw data from these measurements are presented in Figure S2 as normalized intensity at selected q values and in Figure 1d as the mean squared displacement (MSD). It is important to note that MSD is an integrated quantity that includes all dynamics (vibrations, rotations, conformational changes, and diffusion). As such, it cannot provide a description of the motions taking place on its own. It is, however, a valuable tool to detect onset temperatures for particular dynamics.

The temperature trend in the MSD (Figure 1d and Figure S2d) and elastic intensity of the elastin samples (Figure S2a-c) is typical of what is observed for other dry and hydrated proteins, with the addition of the melting of bulk water in the highly hydrated samples. Two onsets are observed at low temperature (Figure S2): one at T ~ 120 K, which is ascribed to the activation of methyl group rotations in proteins[34−38] and the other at T ~ 200 K in the hydrated samples corresponding to the so-called dynamic transition[39] (see also Figure S2). There is a notable effect of water in confining the low temperature motions of the hydrated elastin gel, as evidenced by the fact that the MSDs of both dry and hydrated α-elastin powders are higher than that of the α-elastin hydrogel in the temperature range from T ~ 230 K to ~277 K. The result is similar to what was seen for the β-barrel protein GFP,[40] where water both inside and outside the barrel structure suppresses the motions of the hydrated protein.

The MSD and the elastic intensity do not exhibit a significant change in correspondence with the ITT; a minimal discontinuity is visible only in the elastic intensity at low q (Figure S2c). The ITT coincides with the calorimetric transition observed at 294−297 K in the hydrated α-elastin hydrogel (inset of Figure 2d). The thermogram of the dry α-elastin hydrogel (lyophilized) shows no such peak in this temperature range (Figure 1d), nor does the dry α-elastin powder, confirming that the conformational change requires the presence of water.

An approach based only on the analysis of MSD presents substantial limitations; the analysis of energy-resolved spectra

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with their q and temperature dependence is considerably more informative. These spectra were collected for the α-elastin hydrogel at 280 and 300 K (Figure 2 and Figure S3). The spectra correspond to time scales from hundreds of picoseconds to tens of femtoseconds covering a range of protein motions. In this dynamic window, the spectra of proteins includes the contributions of four processes:[41] (i) methyl group rotations, essentially unaffected by hydration, and which appear as a broad distribution in the hundreds of picoseconds time range (~10−350 ps);[34,36,41] (ii) localized diffusion-like motions of the biomolecule, which are a broad relaxation process enhanced by the presence of water; (iii) fast picosecond dynamics (~0.1−1 ps) that are usually ascribed to the local rattling motions of atoms in a cage formed by neighbor residues and hydration water molecules[38,39]and (iv) low-frequency vibrations. Note that an additional contribution from the tail of the jumps between different conformational states occurring from tens to hundreds of nanoseconds is also possible.[38,39] Both folded proteins and intrinsically disordered proteins (dry or hydrated) appear to exhibit the same classes of motions at these time scales, with similar response to changes in temperature and hydration level.[42]

There is a pronounced increase in the spectra of the α-elastin hydrogel associated with water enhanced, localized diffusive motions below ~1 meV, relative to both the globular protein green fluorescent protein (GFP) and the unfolded protein β-casein (CAS) hydrated powders.[41,42] These motions in the elastin hydrogel show a pronounced maximum around ~0.3 meV at 300 K (~2.2 ps). The increased water content in the elastin hydrogel (h = 1) relative to CAS and GFP hydrated powders (h = 0.4)[41,42] enhances the localized diffusion of the protein which is responsible for the larger scattered intensity. The increase in the scattering intensity is ascribed to water plasticizing the internal motions of the protein and enabling an increase of conformational mobility.[43] These motions should also be sensitive to the hydrophobic collapse and water reorganization at the ITT.

We can extract the geometry of these water enhanced localized diffusive motions by analyzing the dynamic structure factor and extracting the elastic incoherent structure factor (EISF(q)). Briefly, the EISF(q) reflects the ratio of elastic scattering to total scattered intensity at a given momentum transfer, q. The EISF(q) can subsequently be modeled to describe the geometry of the motions and fraction of protons contributing to a given motion. The BASIS spectra for hydrated α-elastin at 280 and 300 K were analyzed considering two processes: methyl group rotations and water enhanced localized diffusion motions (Figure S3a). The water-enhanced localized diffusion was modeled as diffusion in a sphere (inset Figure 2c), and the methyl group rotations were modeled as a three-site jump. The fraction of methyl protons was determined from the amino acid sequence of elastin[44] by taking the fraction of nonexchangeable hydrogen atoms found on methyl groups (P_M = 0.38). There is a larger fraction of methyl protons in α-elastin (P_M = 0.38) relative to GFP (P_M = 0.26[41]) and CAS (P_M = 0.29[42]) accounting for a small source of extra intensity. The radius of the methyl rotations was taken to be R_M = 1.03 Å;[45] these values were used at both temperatures.[41] A more detailed description of this analysis is reported in the Supporting Information. Parameters used in this analysis (full width at half-maximum and amplitude) are reported in Figure S3.

Significantly, this analysis reveals differences in the contribution from the water enhanced localized diffusion below and above the ITT. At 280 K these motions involve 22% (P_{LD} = 0.22) of nonexchangeable hydrogen atoms with amplitude, R_{LD}, of 8.5 Å. At 300 K a higher fraction of hydrogen atoms are participating in the motions (27%), but the motions are occurring over a smaller radius (7.1 Å). This is consistent with the hydrophobic collapse and simultaneous expulsion of water occurring at the ITT. We remark that such amplitude values exceed those seen in other proteins and nucleic acids, and are indicative of a highly flexible and dynamic system both below and above the ITT.[42,46]

We now turn our attention to the collective vibrations of elastin seen in the spectra at energies greater than 1 meV. These low-frequency vibrations, particularly the collective motions known as boson peak (BP), are correlated to the local rigidity of the system.[47−49] These motions are common to glass forming systems and proteins, occurring on the subpicosecond time scale and are thought to be related to acoustical dispersion from nanoscopic regions of differing modulus within the material. In the case of proteins, they are thought to contain contributions from a range of vibrational modes distributed throughout the entire biomolecule.[49−51] Low-frequency collective vibrations have been used to study the dynamical and rigidity properties of systems as a function of temperature, compression, under stress-induced deformation, and uniaxial tensile strain.[33,52−55] Specifically, the BP position, ν_{BP}, can be interpreted as a probe of local rigidity of the system on a few nanometers length scale, with higher frequency correlating to a more rigid system. Recent studies have pointed out a correlation between these motions and the rigidity of the predominant secondary structure of the protein,[48,56,49] with random coils being softer than α-helices, and β-sheets being the most rigid structure. The same analysis also showed that the soft intrinsically disordered protein CAS becomes much stiffer upon calcium binding.[42]

We have applied this approach to the characterization of elastin. Using neutron scattering spectra collected at CNCS with an instrumental resolution of ~20 ps, we characterize ν_{BP} in dry and hydrated powders, in the dry gel at 300 K, as well as in the hydrated gels at 280 and 300 K. These spectra are presented in Figure 3a using the spectral density formalism to highlight the two contributions, a quasielastic scattering component at low energy and the boson peak. The data were fit over the same energy range used previously for other proteins [0.6−6 meV],[48,56] in order to directly compare the obtained boson peak frequencies to prior literature (details of the fitting procedure can be found in the Supporting Information). The ν_{BP} values indicate that elastin is a soft protein,[48,56] compatibly with disordered structures. Such low stiffness is in line with a highly dynamic system and reconciles with the definition of elasticity, as the ability to be deformed reversibly to large strains with little force and without loss of energy.[57] This result is also consistent with the high hydrophobicity of elastin and low number/strength of hydrogen bonding sites per residue, when compared to most proteins.[48,56,58] MD simulations obtained for the elastin-like peptide, ELP, $(VPGVG)_{18}$ at high temperature support our results: only ~10−15% of the ELP's main-chain polar groups are involved in main-chain/main-chain hydrogen bonds.[18] This value is significantly lower than the average of 50% registered for globular proteins.[59] Additionally, our experimental data show that the trends observed for other globular and unfolded proteins[42,48,56,60] are also valid for elastin materials, with progressive stiffening associated with the presence of hydration

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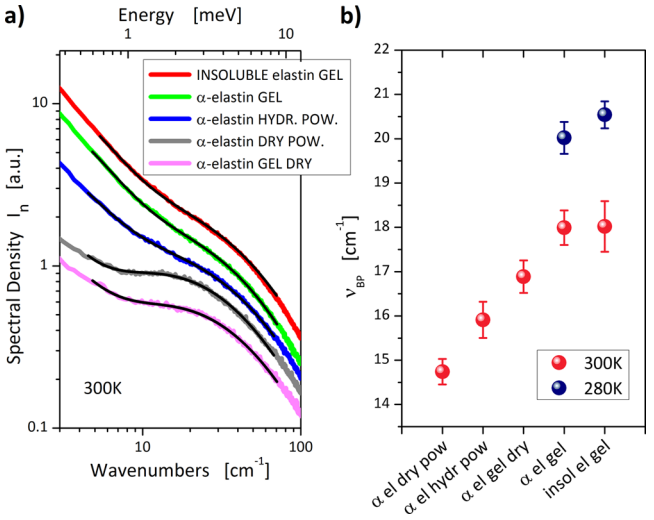

Figure 3. Rigidity of elastin: soft disordered biomaterials. (a) Boson peak spectra of α- and insoluble elastin samples presented in the spectral density formalism at 300 K. Black lines represent the total fit obtained using eq 5 in the Supporting Information. Spectra obtained for α- and insoluble elastin hydrogels at 280 K are shown in Figure S4. (b) Boson Peak frequencies obtained from the fit of the neutron spectra at 300 K (red) and 280 K (blue). Values are reported in order of increasing ν_{BP}.

water, addition of salt, cross-linking and temperature decrease (Figure 3b). Finally, we observed that α-elastin and insoluble elastin hydrogels have comparable rigidities at both temperatures (Figure 3b).

We then examined the low-frequency vibrations under mechanical elongation to investigate the origin of elastin's elasticity. Neutron scattering spectra were collected as a function of uniaxial elongation in the range from 0.03 to 47 meV to interrogate the local elasticity of elastin gels hydrated with D_2O through the low frequency vibrations, including the boson peak. The neutron scattering experiments were designed to exclusively probe the dynamics of the elastin protein in the hydrogel at 300 K (Figure 4). Spectra were collected for α-elastin at strains of 0% and 48% (Figure 4b) and insoluble elastin from 0% to 70% strain (Figure 4c). Both sets of data show that the position and spectral shape of the low frequency vibrations of elastin are unperturbed at physiological strains up to 70%. This shows that the local (few nanometers) rigidity and associated collective vibrations of elastin are not affected by uniaxial elongation above the ITT. Elastin molecules remain disordered, though more extended, during elongation and do not crystallize upon stretching in this regime.[10] Our experimental result is consistent with the notion that elastin hydrogels behave like nearly ideal elastomers. Comparable observations of unperturbed collective vibrations in silkworm silk[55] upon uniaxial tensile strain have also been reported. The mechanical properties of these biomaterials can be explained in terms of a model of entropic elasticity.[55] According to this description, the elastin chains reorient along the applied stress direction, which is confirmed by Raman experiments.[61] The elastic restoring force then originates from a reduction in the number of polypeptide chain configurations and the associated reduction in the configurational entropy.

This interpretation is consistent with the classic understanding of rubber elasticity.[16,8,10] This considers elastin as a network of polypeptide chains randomly distributed and swollen with an equal weight of water. These authors attribute the origin of the elastic restoring force to the displacement from this position of highest entropy. Thermoelastic measurements[9] and MD simulations[18,23] of elastin like peptides have been offered in support of this hypothesis. Single molecule force spectroscopy on the monomeric precursor of elastin, tropoelastin, recently found a persistence length much smaller than the length of the molecule, also indicating entropic elasticity.[62] Similar single molecule experiments on tropocollagen also suggest entropic elasticity at small deformation.[63] Only at larger deformations does energetic elasticity become the dominant contribution with the breaking of hydrogen bonds, deformation of covalent bonds in the protein backbone, and eventually molecular fracture.

A different interpretation of the elasticity properties of elastin peptides assumes a well-defined secondary structure, distortion from which generates a restoring force based on a librational entropy mechanism.[11,12] These works claim experimental support from both CD and NMR.[13] However, our experiments

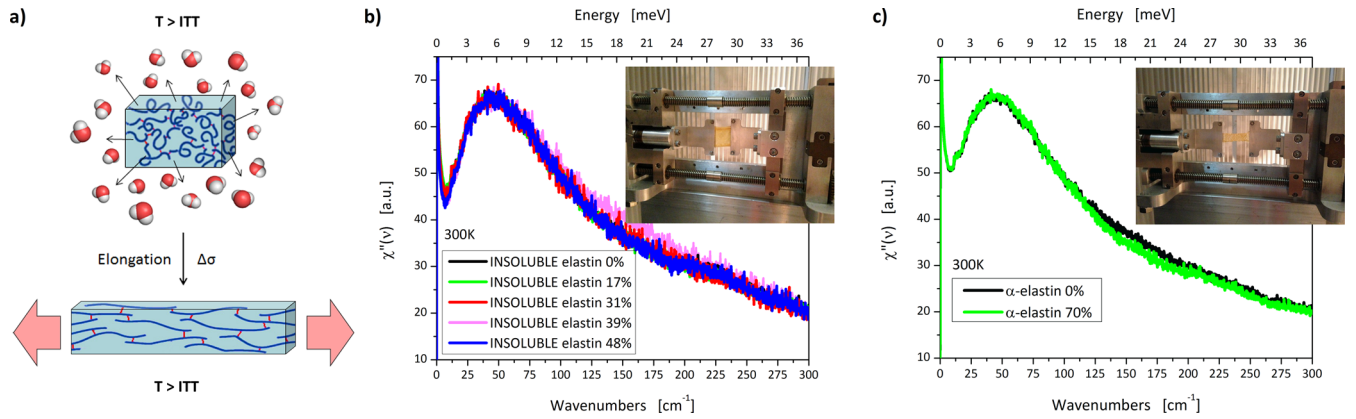

Figure 4. Collective vibrations of elastin gels under strain. (a) Scheme of elongation of elastin hydrogel above T_t. Susceptibility spectra of all the low-frequency vibrations of insoluble elastin (b) and α- elastin (c) hydrogels under uniaxial strain (300 K). Insets: pictures of the insoluble (b) and α-elastin (c) hydrogels mounted on the cell of controlled elongation specially designed at SNS. The spectra were scaled to the maximum height without any additional manipulation. Insets show pictures of both kinds of hydrogels samples mounted on the elongation cell and placed inside the CNCS spectrometer. The static structure factor $S(Q,0)$, extracted from the elastic line of the neutron scattering data, was used to verify that there was no loss of water during the measurements (Figure S5 in the Supporting Information).

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would be sensitive to changes in the secondary structure upon elongation,[42,48,49,56,60] and we do not observe the predicted variations in local rigidity as a function of strain. This elastomeric behavior in elastin requires the presence of water. Water acts to plasticize the polypeptide chains and increase the conformational mobility within the network. The fact that the hydration of elastin is predominantly hydrophobic in nature suggests that the reorganization of the water network upon elongation does not require extensive making/breaking of hydrogen bonds with the protein. This result is consistent with the mainly entropic origin of the elasticity in elastin.

In conclusion, this work provides detailed molecular level information related to the ITT and the superb elastomeric properties of elastin hydrogels. Both of these remarkable features are consistent with a similar mechanism (Figures 2a and 4a) of entropic origin, connected to hydrophobic hydration. Water has an important role plasticizing the dynamics of elastin, a very soft and flexible material on the nanometer length scale. In this way, water enables the protein to sample a larger number of conformational states. In the case of the ITT, the data show a reduction in the length scale of localized diffusion motions (from 8.5 Å to ∼7.1 Å) as a result of the hydrophobic collapse and expulsion of water molecules from the nonpolar surfaces at the transition temperature. This reflects the change in structure to a more compact and entropically favorable state above the ITT. It has been suggested that a return to this entropically favorable state acts as the restoring force at the heart of elastin's elasticity. Neutron scattering measurements of the low-frequency vibrations as a function of elongation have shown that the local rigidity of the protein is unaltered upon elongation below strains of 70%, which is consistent with a model of entropic elasticity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.5b01890.

Materials and methods, 1 table describing the scattering cross sections of elastin samples; and five figures detailing the temperature dependence of the elastic intensity; EISF(q) analysis; the temperature dependence of the boson peak; and static structure factor for measurements under elongation (PDF)

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Notes

The authors declare no competing financial interest.

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