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The Coupled Bio-Chemo-Electro-Mechanical Behavior of Glucose Exposed Arterial Elastin

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Abstract

Elastin, the principle protein component of the elastic fiber, is a critical extracellular matrix (ECM) component of the arterial wall providing structural resilience and biological signaling essential in vascular morphogenesis and maintenance of mechanical homeostasis. Pathogenesis of many cardiovascular diseases have been associated with alterations of elastin. As a long-lived ECM protein that is deposited and organized before adulthood, elastic fibers can suffer from cumulative effects of biochemical exposure encountered during aging and/or disease, which greatly compromise their mechanical function. This review article covers findings from recent studies of the mechanical and structural contribution of elastin to vascular function, and the effects of biochemical degradation. Results from diverse experimental methods including tissue-level mechanical characterization, fiber-level nonlinear optical imaging, piezoelectric force microscopy, and nuclear magnetic resonance are reviewed. The intriguing coupled bio-chemo-electro-mechanical behavior of elastin calls for a multi-scale and multi-physical understanding of ECM mechanics and mechanobiology in vascular remodeling.

I. Introduction

Elastin provides many tissues with remarkable resilience and longevity. The human aorta is comprised of approximately 47% elastic fibers and undergoes billions of stretch cycles in the course of one's lifetime (Starcher and Galione 1976). Elasticity is crucial for the aorta to accommodate the pulsatile blood flow. Elastic fibers consist of an inner crosslinked elastin core surrounded by a mantle of fibrillin-rich microfibrils (Mecham 2008). In elastic arteries such as the aorta, elastic fibers form thick concentric fenestrated layers of elastic laminae, with inter-laminar connecting fibers distributed radially through the vessel wall (O'Connell et al. 2008). Each elastic lamina alternates with a layer of smooth muscle cells, collagen fibers, and together organizes into a laminar unit, which is considered as the functional unit of the vessel wall (Wolinsky and Glagov 1967) (Figure 1). Collagen fibers are designed to provide structural integrity and mechanical properties at higher strains. The triple helix

structure of collagen molecules bundle together to form collagen fibrils. Collagen has a half-time of ~22 days in arteries (Humphrey, Dufresne, and Schwartz. 2014), while elastin has a half-life of nearly 70 years and is deposited in early childhood. Elastic fibers are expected to provide elasticity to the tissue through the lifetime of the individual (Sherratt 2009).

There are many biochemical alterations in the ECM which are associated with aging and disease, glycation, lipoxidation, and/or oxidization—each alters the long-lasting ECM structural proteins (Sell and Monnier 2012; Zarkovic et al. 2015). Arterial elastin is an extremely hydrophobic protein, which makes it an attractive site for the deposition of hydrophobic ligands such as lipids that may potentially alter elastin's mobility (Lillie and Gosline 2002). Non-enzymatic glycation, the reaction between glucose and the ECM proteins (Winlove et al. 1996; Winlove and Parker 1990), is enhanced in diabetic patients. It is one of the main mechanisms of ageing of the long-lived ECM protein, and has been shown to correlate with the severity of diabetic complications (Vishwanath et al. 1986; Vlassara, Brownlee, and Cerami 1986), and to accelerate age associated stiffening of arteries in diabetic patients (Cameron et al. 2003).

Despite the documented biochemical modifications of the ECM and their association with cardiovascular disease (CVD), the molecular-level alterations and their effect on the biomechanical functionality of the ECM remains poorly understood. Additionally, little is known about the important pathophysiological effects of the coupled biochemical and mechanical ECM remodeling on the cardiovascular system – this knowledge is integral to understanding the ECM behavior in living biological systems. In this work, we report on a review of our recent studies relating to the contribution of elastin to vascular mechanics with a focus on understanding the constitutional ECM structure, function, and the effect of excessive glucose exposure. Ferroelectric and nuclear magnetic resonance spectroscopic studies revealed molecular structure modifications, and the intriguing coupled bio-chemo-electro-mechanical behavior of elastin. To this end, Section II focuses on the structural and functional contribution of elastin to vascular mechanics, and alterations in elastin function with glucose exposure; Section III focuses on the electromechanical properties of elastin reveals by ferroelectric studies; and Sections IV and V focuses on understanding the molecular structural and dynamical alterations of elastin due to glucose exposure by nuclear magnetic resonance (NMR) and molecular dynamics (MD) studies.

II. Contribution of elastin to vascular mechanics

II.1 Sequential engagement of ECM constituents in arterial mechanics

The structural and mechanobiological interrelations between elastin and collagen, the two primary load-bearing components in the arterial wall, are important for properly functioning arteries. To understand the elastic and viscoelastic properties of elastin, purified elastin network was used for mechanical testing (Gundiah, B Ratcliffe, and A Pruitt 2007; Lillie and Gosline 2002, 2007; Lu et al. 2004; Zou and Zhang 2009, 2011). Elastin was shown to possess an anisotropic stress-strain response that is comparable to the intact arteries (Zou and Zhang 2009). Moreover, elastin is responsible for the linear elastic response of blood vessels at lower strains. While at higher strains, the mechanical response of the aorta

becomes much more nonlinear and stiffer in both directions due to the strain stiffening and the engagement of collagen fibers.

Advances in optical methods and image processing techniques have made it possible to quantify the architecture of the ECM components of soft tissues at different scales (Holzapfel 2008). Using a multi-photon microscope, second harmonic generation and two-photon fluorescence signals have been simultaneously captured from arterial collagen and elastin (Chow et al. 2014; Fata et al. 2013; Hill et al. 2012; Wan, Dixon, and Gleason 2012; Zeinali-Davarani et al. 2013). These experimental methods allow for an in-depth understanding of the relationships between arterial ECM mechanics, microstructure, and mechanobiology. Chow et al. (Chow et al. 2014) studied the structural changes in elastin and collagen fiber organization, realignment, and recruitment by coupling mechanical loading and multi-photon imaging to capture real time changes to the microstructure during biaxial deformation, which was applied using a custom-designed stretcher (Figure 2). The medial collagen shows a preferred circumferential distribution, however the adventitial fiber family distribution are obscured by their waviness at no or low mechanical strains. Collagen fibers in both layers exhibit significant realignment in response to unequal biaxial loading. The elastic fibers, however, are much more uniformly distributed and remained relatively unchanged during loading. This study provided new quantitative evidence on the sequential engagement of ECM constituents in response to mechanical loading. It was shown that the adventitial collagen exists as large wavy bundles of fibers that exhibit delayed fiber engagement after 20% tissue strain. The medial collagen is engaged throughout the stretching process, and prominent elastic fiber engagement is observed up to 20% strain, after which the engagement plateaus. This study also suggested that the elastic fibers are under tension and impart an intrinsic compressive stress on collagen. Additionally, there is an intrinsic interrelation between elastin and collagen fibers, that determines the mechanics of arteries and may carry important implications to vascular homeostasis and mechanobiology.

II.2 Progression of mechanical stages with gradual elastin degradation

Elastin is a very stable protein with low turnover and little degradation in healthy individuals (Robert, Robert, and Fülöp 2008; Sherratt 2009). However, injury, aging, and/or disease can lead to a reduction in the functional properties of elastin and impair tissue function (Carmo et al. 2002; Lakatta et al. 1987). For example, a 90% reduction in elastin is reported in abdominal aortic aneurysm (AAA) specimens compared to non-aneurysmal abdominal tissue (Carmo et al. 2002). As a result of these structural changes, human aortic tissue with aneurysm shows increased elastic modulus and anisotropy compared to healthy tissue (Vande Geest, Sacks, and Vorp 2006; Humphrey and Taylor 2008; Matsumoto et al. 2009). With a goal of understanding the relationship between the mechanical function and the structural/biological composition of an artery, simple chemical digestion models of elastin degradation have been used to investigate the progressive changes in arterial mechanical properties (Chow, Choi, et al. 2013; Chow, Mondonedo, et al. 2013; Zeinali-Davarani et al. 2013). Studies by Chow et al. (Chow, Mondonedo, et al. 2013) suggested that gradual elastin degradation in the artery can lead to a progression through different mechanical properties characterized by four distinct mechanical stages (Figure 3). Using multi-photon microscopy,

the authors showed that degradation of elastin significantly reduces the undulation of collagen fibers resulting in an increase in the artery diameter. While being less undulated, collagen fibers are ready to be recruited upon mechanical loading resulting in a less compliant and much stiffer arterial wall (Zeinali-Davarani et al. 2013). The study by Chow et al. (Chow, Mondonedo, et al. 2013) also revealed the potential existence of an important transition stage (~40% elastin remains) may lead to mechanical instability, i.e., large increases in artery diameter with small mechanical perturbation contribute to arterial dilation during aneurysm formation. These studies explored how changes in the microstructure of elastin affect the mechanics of arteries and helped further the understanding of the role of elastin in arterial wall mechanics.

II.3 Biochemical glucose exposure results in stiffened and viscoelastic elastin

Elastin is a long-lived ECM protein and it can suffer from cumulative effects of biochemical damage. *In vitro* studies have shown that glucose treatments stiffens arterial elastin (Winlove et al. 1996), increases the storage and loss modulus (Lillie and Gosline 1996), and also changes the viscoelastic stress relaxation behavior of elastin (Zou and Zhang 2011). Elastin was found to become markedly stiffer and more viscoelastic with excessive glucose exposure (Wang et al. 2015; Zou and Zhang 2012). Mechanical testing results (Figure 4) show that the stiffness of elastin increases gradually with glucose treatment. In addition, glucose treated elastin exhibits a large hysteresis upon loading and unloading compared to control samples. Moreover, the amount of hysteresis also increases with treatment times. Transmission electron microscopy (TEM) studies revealed no obvious structural changes in elastin due to glucose treatment, suggesting molecular level structural modifications due to the interactions between elastin and glucose (Wang et al. 2015).

The marked stiffening of elastin in glucose may have important medical and physiological implications. For instance, the increased viscous damping may have detrimental effects on the cardiac function; arteries will lose energy upon moving the blood further down the arterial tree during diastole, and higher systolic pressures are required to generate flow in stiffer and dampened arteries. However, little is known about the important pathophysiological effects of the coupled biochemical and mechanical changes on the cardiovascular system.

III. Bio-chemo-electro-mechanical behavior revealed by piezoresponse force microscopy (PFM)

We have recently demonstrated that arterial elastin responds mechanically to an electric stimulus. Using a piezoresponse force microscope, our study shows that the polarity of elastin is switchable by an electrical field. Moreover, our study revealed that glucose can freeze the internal asymmetric polar structures of elastin and make it more difficult to switch, and that there is strong correlation between early stage atherosclerosis and electromechanical coupling of aorta. As elastin is present in all connective tissues of vertebrates, the inhibition of ferroelectric switching by glucose, and the correlation between aortic hardening and electromechanical coupling, may have profound implications for understanding the ageing process and numerous degenerative diseases.

III.1 Piezoelectricity and ferroelectricity of elastin

It is well known that many biological tissues are piezoelectric (Li et al. 2013), exhibiting linear coupling between deformation and an electric field. Piezoelectricity is thought to be a fundamental property of biological tissues (Shamos 1967), possessing important physiological significance (Lang 2000). In blood vessel walls, piezoelectricity was first reported by Fukada and Hara through a macroscopic measurement (Fukada and Hara 1969), and the effect was recently revisited using a piezoresponse force microscopy (Liu et al. 2012). This atomic force microscopy (AFM) based technique applies an AC voltage to the sample through a conductive probe, exciting piezoelectric vibration of the sample that can be measured with picometer sensitivity and nanoscale resolution (Li et al. 2015). In addition to confirming piezoelectric effect in aortic walls, we also observed the reversal of polar direction by an external electric field, the so-called ferroelectric switching that was speculated to exist in biological tissues (Lang 2000), but not experimentally verified before.

The observation of ferroelectric switching in the aortic wall is interesting, and raises important questions regarding its origin and significance. Subsequent studies revealed that the phenomenon originates in elastin, as collagen was found to be non-switchable (Liu et al. 2013). As shown in Figure 5, the piezoresponse is clearly visible in a $1\mu\text{m}^2$ mapping of elastin consisting of three fibers, and the polarity of such response can be reversed by a DC voltage, as indicated by the phase-voltage hysteresis and amplitude-voltage butterfly loops. Specifically, the phase of the piezoelectric force microscopy (PFM) response is observed to be reversed by 180° , which is a signature of ferroelectric switching found only in elastin, but not in collagen, even though collagen is also piezoelectric.

Our subsequent PFM investigation showed that the ferroelectric switching is observable in tropoelastin. Molecular dynamics simulations revealed that tropoelastin exhibits a large electric dipole moment that may be switched by external electric field (Liu et al. 2014). As tropoelastin monomers are cross-linked into elastin in a head-to-tail manner (Yeo et al. 2012), both dipole moments and their switching are preserved in elastin—this study shed considerable light into the molecular mechanism of this ferroelectric effect. Furthermore, macroscopic ferroelectric switching is also observed in elastin through pyroelectric measurements, by heating the sample through a laser, and thus inducing pyroelectric currents that depends on the dipole direction. This series of studies thus establish ferroelectric switching and its molecular mechanism in elastin.

III.2 Suppression of ferroelectric switching in elastin by glucose

What is the significance of piezoelectricity and ferroelectricity of elastin? A number of investigations offer us some clues. In one study, we found that ferroelectric switching is largely suppressed by *in vitro* glucose treatment (Liu et al. 2013), as seen in Figure 6. The switching spectroscopy PFM mapping of the remnant piezoresponse on a grid of 32×32 points over $5\times 5\mu\text{m}^2$ area for glucose-treated elastin is shown in Figure 6(b), in which large areas marked as blue are identified that shows no switching characteristics, accounting for 30.7% of total points probed. This is better illustrated in Figure 6(d) and (e), where the points outside of blue area shows clear hysteresis and butterfly loops, while points inside the blue area show very small variation in phase and rather irregular amplitude loops. In fact,

even for points outside of the blue areas, the switching characteristics are also substantially altered by glucose treatment, with the amplitude-voltage butterfly loops becoming highly asymmetric. This is confirmed by switching spectroscopy PFM mapping of nucleation bias shown in Figure 6(c), which ranges from -20 to -38 V, much larger than those observed in untreated elastin. This observation suggested that glucose treatment seems to freeze the internal asymmetric polar structures of elastin via glycation, making it more difficult to switch. The difference is statistically significant, as shown by Figure 6(f), as the the percentage of the no switching points ($49.68\% \pm 5.54\%$) in the glucose-treated elastin is significantly higher than the untreated elastin (0%) ($p < 0.05$).

To further understand the suppression of ferroelectricity in elastin by glucose treatment, we also carried out detailed PFM mapping for glucose-treated elastin. These measurements showed that points within high PFM response areas exhibit clear yet notably asymmetric switching characteristics, while those within low response areas are largely non-switchable. Furthermore, the statistical distribution of PFM amplitude over four $1\mu\text{m} \times 1\mu\text{m}$ areas in respective samples reveal a large spike at very weak PFM amplitude, which is clearly absent in untreated elastin samples. These observations suggest that glucose also reduces the piezoelectric response of elastin substantially, which could be related to crosslinking that stiffens elastic fibers and reduces their piezoelectricity. Indeed, we also found increase of tangent modulus in glucose treated elastin (Wang et al. 2015; Zou and Zhang 2012). The structural and functional alterations of elastin with glucose exposure may have important physiological implications. The polarization in elastin may help in regulating proliferation and organization of vascular smooth muscle cells and contribute to arterial morphogenesis (Athenstaedt 1974). Additionally, these changes could have implications for problems found in the elderly with a lifetime of exposure to glucose, and to younger individuals with high sugar diet.

III.3 Correlation between atherosclerosis and electromechanical coupling of aorta

Another interesting observation is the strong correlation between early stage atherosclerosis and electromechanical coupling of aorta was recently reported (Liu et al. 2016). Atherosclerosis is the underlying cause of cardiovascular diseases (Lusis 2000), and the existing imaging methods are not capable of detecting the early stage of atherosclerosis development due to their limited spatial resolution (Nakashima, Wight, and Sueishi 2008). This motivated us to examine the effect of atherosclerosis on the electromechanical coupling of the aorta, which may provide us an alternative imaging method with high sensitivity and spatial resolution (Halperin et al. 2004). To this end, we examined aorta samples from two types of mice fed by either a chow diet or a high-fat diet. Since plaque was found to develop in Apolipoprotein E (APoE) $^{-/-}$ mice fed with high-fat diet, aortic wall samples both on the spot of plaque and away from it were examined. Altogether, this results in four groups of samples as follows: group 1 and 2 were taken from C57BL/6J wide type mouse and APoE $^{-/-}$ mouse fed with normal diet, respectively, and group 3 and 4 were taken from APoE $^{-/-}$ mouse fed with high fat diet in the plaque-free region, and on the spot of plaque region, respectively. These samples are thought to correspond to different stage of atherosclerosis development, and we hoped to detect it via electromechanical coupling.

The results of corrected vertical PFM amplitude of these four different samples are shown in Figure 7a, and a clear correlation is observed: the PFM amplitude increases from group 1 to group 4 in a monotonic manner, which is clearly exhibited by the histogram distributions in Figure 7b. By analyzing 12 samples from each group of samples, this trend is also confirmed to be statistically significant. Furthermore, it was observed that as atherosclerosis develops, the coercive voltage that is necessary to switch the polarity of the sample increases, though on the spot of plaque the coercive voltage actually drops, which is believed to be caused by deposition of lipids or small polar molecules on plaque. These set of data thus suggest a strong correlation between atherosclerosis development and electromechanical response of aortic wall, and that electromechanical response is a much more sensitive and reliable measure for atherosclerosis development.

To make sure that the contrasts seen in Figure 7 have statistical significance, we analyzed 12 samples from each group of tissues, yielding 48 mappings of corrected PFM amplitude as well as 48 mappings of resonant frequency that correlates with sample stiffness. Both peak and mean values of corrected amplitude and resonant frequency for each groups of samples are summarized in Table 1, and statistical analysis showed that the peak value and mean value of corrected PFM amplitude were significantly different when comparing with each group ($p < 0.05$). As for the peak value of resonant frequency, the significant differences exist between group 1 and group 3, group 1 and group 4, group 2 and group 4, and group 3 and group 4 ($p < 0.05$). For the mean value of resonant frequency, the significant differences exist between group 2 and group 4, as well as between group 3 and group 4. These data suggested that electromechanical response is a much more sensitive and reliable measure that correlates with the atherosclerosis development, while stiffness, or resonant frequency, is less sensitive, especially during the early stage of atherosclerosis development.

Furthermore, clear contrast in coercive voltage, the threshold voltage that is necessary to switch the polarity of the sample, is observed among these four different groups of samples. With atherosclerosis development, the coercive voltage increases, until the plaque develops, on top of which the coercive voltage drops substantially, again suggesting a strong correlation between electromechanical responses and atherosclerosis development. The polarity of the sample becomes increasingly more difficult to switch, until the plaque is developed, wherein the deposition of lipids or small polar molecules makes it easier to switch. As such, the coercive voltage offers additional measure that correlates with atherosclerosis development.

IV. Structural and dynamical alterations of elastin due to glucose exposure by nuclear magnetic resonance (NMR)

Elastin's precursor, tropoelastin, is approximately 72kDa in size and is cross-linked by desmosine (formed by from three allysyl side chains and one unaltered lysyl side chain) or isodemsosine (formed by four lysine residues). This infinitely sized molecule exhibits a large number of degeneracies in a one-dimensional natural abundance ^{13}C solid-state NMR experiment. While solid state ^{13}C NMR has emerged as powerful tool for studying the structure and conformational dynamics of a broad range of biological polymers (McDermott

and Polenova 2010), determining the precise structure of elastin, or its precursor tropoelastin, remains a formidable challenge with current experimental methods.

Early work by Fleming and coworkers (Fleming, Sullivan, and Torchia 1980), involving $[1-^{13}\text{C}]$ valine, $[1-^{13}\text{C}]$ alanine, and $[1-^{13}\text{C}]$ -lysine labeled chick aortic elastin, highlighted the importance of a solvent in the backbone dynamics and provided measurements of chain mobility by linewidth measurements. More recent two-dimensional ^1H - ^{15}N experiments have been performed, with success, to characterize mobile domains of elastin using liquid state approaches (Ohgo et al. 2005). At present, these two dimensional experiments are not feasible in the solid state without site-specific labels. However, numerous works have been reported on the structure and dynamics of repeating elastin peptides, which have often focused on hydrophobic regions such as VPGVG (Hong et al. 2003; Ohgo et al. 2005; Pometun, Chekmenev, and Wittebort 2004; Yao and Hong 2004), LGGVG (Kumashiro et al. 2003), and VGVAPG (Floquet et al. 2004). The Keeley group has also performed numerous experiments giving structural insights to elastin derived peptides (Miao et al. 2013; Muiznieks et al. 2015; Reichheld et al. 2014).

We have recently studied the structure and dynamics of unlabeled aortic elastin by excessive *in vitro* glucose exposure (Silverstein et al. 2015), and have observed several structural and dynamical alterations. An increase in the signal intensity following glucose exposure is observed in our ^1H - ^{13}C cross polarization spectra; the glucose treated sample cross polarizes significantly better than the untreated samples pointing to a more rigid backbone. These changes are observed to occur throughout the entire protein. Second, glucose treatment induces significant differences in spectral resolution – the untreated sample exhibits narrower spectra than the glucose treated sample, indicating a higher degree of structural heterogeneity in the glucose treated sample in comparison with the untreated sample. Third, chemical shifts differences at ~17ppm and ~52ppm (corresponding to $\text{C}\beta$ and $\text{C}\alpha$ alanine peaks) in the untreated and glucose treated samples appear to be suggestive of higher α -helical like secondary structures following glucose exposure. Lastly, the signals observed in Figure 8 between 70 to 80 ppm and approximately at 62 ppm in the glucose treated sample, resulted from residual glucose present in the tissue. The observation of cross-polarized glucose peaks would indicate that the glucose exhibits anisotropic motion and/or slow motion and may be a signature of glucose bonding to the protein backbone.

Our measurements of protein dynamics by NMR studies reveals that the correlation times of many amino acids ($\text{C}\alpha$ glycine, $\text{C}\beta$ valine, $\text{C}\beta$ proline, as well as the $\text{C}\beta$ and $\text{C}\gamma$ alanine moieties) all appear larger for the glucose treated sample than for the untreated sample (see Table 2). The correlation times were determined from the ratio of two relaxation times at different spin locking intensities, and provide detailed information regarding dynamical fluctuations of the ^1H - ^{13}C inter-nuclear vector fluctuations at hydrophobic and hydrophilic sites of elastin. The measurements indicate that changes following exposure to glucose modify the dynamical behavior of elastin not at one site, but throughout the entire protein. The larger correlation times indicate a reduced dynamical behavior of the protein backbone following glucose exposure, and results in altered entropic and energetic contributions to the elastomeric force resulting in a stiffening of the elastin backbone (results are corroborated by molecular dynamics simulations in the following section).

The mechanistic properties of elastin are directly related to its structure and dynamics. Elastin is a special ECM protein whose elasticity appears to be driven by an entropic force that is related to its dynamical and/or conformational alterations. Our previous study (Silverstein et al. 2015) demonstrated that *in vitro* non-enzymatic glycation induces important structural, dynamical, and functional alterations in elastin. The observed stiffening and increased viscous dampening of elastin is consistent with NMR data on modified structure and reduced conformational mobility.

V. MD simulations to investigate the source of stiffening in glucose-exposed elastin

The previous section summarized some of our recent NMR chemical shift and relaxation measurements which pointed to dynamical and structural changes of elastin exposed to glucose. The measured chemical shifts and relaxation times on unlabeled samples represent average quantities (e.g. all glycine residues). Without domain and site specific labels (e.g. through bacterial expression) a complete structure and the interaction with a solvent (e.g. glucose) is likely not possible to obtain even with modern magnetic resonance methods. Therefore, a piecewise study of hydrophobic and/or hydrophilic building blocks of elastin based on molecular dynamics simulations is useful for obtaining thermodynamic, structural, and dynamical information. The primary structure of the monomer of bovine nuchal ligament elastin, tropoelastin, has been reported previously (Debelle and Tamburro 1999). The complex structure includes polyalanine motifs, such as the pentamer AAAAA which repeats 14 times, as well segments such as VPGVG which repeat 9 times in human elastin (He et al. 2007). The pentamer VPGVG has been shown to behave as an ideal elastomer in previous experimental studies, including AFM (Urry et al. 2002). We therefore performed molecular dynamics simulations of these peptides with and without glucose to gain insight into dynamical and structural alterations which may influence macroscopic behavior.

In general, the elastomeric restoring force (f_{tot}) of any elastomeric material may be characterized by changes in the energy ($f_e = dU/dr$) and/or entropy upon being stretched ($f_s = -TdS/dr$), $f_{\text{tot}} = f_s + f_e$. Energy changes may result from alterations in peptide-peptide or peptide-solvent interactions. Two well known models that have been put forth in the community of elastin researchers are the so-called librational entropy mechanism and the sliding beta turn. In the librational entropy mechanism suspended segments of the form GVGVP undergo oscillations of pairwise torsion angles which dampen upon extension (Urry et al. 2002). In the sliding beta turn model, however, labile type II beta turns for GXGGX type sequences may exhibit hydrogen bonds, which interconvert under strain, giving rise to conformational changes (Tamburro et al. 1990). It is worth noting that while both models refer to different segments of elastin, both models assume a change in entropy giving rise to the elastomeric force, while the energetic term is assumed to be negligible.

Early molecular dynamics simulations of repeating segments of elastin have indeed shown that changes in entropy upon extension far exceed any energetic contributions. Chang and Urry (Chang and Urry 1989) showed that the entropic contribution (f_s) of the elastin mimetic peptide VPGVG are much larger the energetic term (f_e). Wasserman and Salemmé also

performed simulations on (VPGVG)₁₈ in water (Wasserman and Salemme 1990). In their work, conformational entropy reduces upon extension. Additionally, they showed that hydrophobic interactions play a key role at small extensions, whereas the librational mechanism contributes to the elastic recoil force at longer extensions.

A generalized method for determining the entropy of a macromolecule involves considering atomic fluctuations. In this formalism, termed the quasiharmonic approach, the entropy (*S*) is determined by

$$S = k_B \sum_i^{3n-6} \frac{\hbar\omega_i/k_B T}{e^{\hbar\omega_i/k_B T} - 1} - \ln \left(1 - e^{-\frac{\hbar\omega_i}{k_B T}} \right) \quad (1)$$

where ω_i are computed from the eigenvalues of the mass-weighted covariance matrix of the coordinate fluctuations (Andricioaei and Karplus 2001). The above expression is familiar from the field of statistical thermodynamics as it represents the entropy of a harmonic oscillator at temperature *T*. Previous studies have applied this approach to study the entropy of macromolecules, e.g. DNA (Harris et al. 2007). We have performed a number of simulations of short repeats of elastin, including the segments VPGVG and LGGVG, to probe the entropic changes upon elongation (Huang et al. 2012). Simulations were performed in relaxed and strained states to determine the entropic force ($f_s = -T dS/dr$). Using the above expression, it was shown that the peptide entropy indeed decreases upon being stretched. The entropy of the strained [VPGVG]₃ peptide was 1.1 kJ/mol K, and a similar value for the strained [LGGVG]₃ peptide of 1.3 kJ/mol K was computed. The values determined via the quasi-harmonic approach appear to be similar to the entropy computed by a previous molecular dynamics study of [VPGVG]₁₈; the average entropy in their computation for a similarly sized peptide is ~ 0.8 kJ/mol K in good agreement to what was obtained in our study (Wasserman and Salemme 1990). Using the change in radius of gyration we estimated the entropic recoil force for the two elastin repeats and found we recoil force (f_s) is 993 kJ/mol nm for [LGGVG]₃ and 780 kJ/mol nm for [VPGVG]₃.

We have performed further simulations to probe the effects of a solvent (other than water) on the entropic and energetic contributions of short elastin repeats. Experiments have been performed to study the effect of glucose on the repeating segment VPGVG and a polyalanine domain, AAAAA. Details regarding the molecular dynamics simulations have been published previously (Silverstein et al. 2015). Our simulation findings indicate that the ratio of the energetic contribution to the total retractive force increases for the peptide VPGVG in glucose in comparison to water. Referring to Table 3 we find that the ratio f_e/f_{tot} is -0.2 in water, but increases to 0.37 when this peptide is placed in glucose. The total retractive force also increases from 8.0 kJ/(mol Å) in water to 10.0 kJ/(mol Å) when the peptide is placed in glucose. The source of the increase in the energetic contribution of the retractive force arises from a peptide-glucose interaction energy. For this repeat, a reduction in the root mean square fluctuations was observed which contributes to the entropy evaluated by Equation (1) above. The reduction in peptide backbone motion in glucose appears to correlate well with our NMR measurements; we observed a smaller ¹H-¹³C cross

polarization efficiency, and a larger correlation time for many of the ^1H - ^{13}C internuclear vector fluctuations. The ratio f_e/f_{tot} is observed to increase slightly from 0.23 in water to 0.20 in glucose for the pentamer. However, the changes in the total retractive force also reduce from 29.3 kJ/(mol Å). These findings would suggest that the marked increase in stiffness of elastin may not arise from changes in the polyaniline domains, but more so from changes in segments such as VPGVG.

For the alanine pentamer we find that the presence of glucose results in an increase in the alpha helical signature, (Figure 9 highlights the Ramachandran maps of the alanine pentamer in water and in 2M glucose) which correlates well with the observations made through our ^1H - ^{13}C cross polarization measurements. Evidently, the changes in the polyaniline domains do not appear to be a major contributor to the increased stiffness of glucose exposed elastin.

VI. Challenges and future directions

The mechanical, structural, and dynamical studies of elastin reveal the interesting coupled bio-chemo-electro-mechanical behavior, which could have profound implications for understanding the ageing process and numerous degenerative diseases. Important modifications due to changes in the local biochemical environment, and their effect on tissue mechanics are not fully understood. This lack of understanding is likely to be correlated with the understudied ECM mechanics in diseases and the lack of experimental techniques to reveal the structural and molecular mechanisms in ECM remodeling—this knowledge is integral to understanding the performance of elastin in living biological systems. It is important to note that the ECM molecules are highly organized, and that minor alterations such as a single amino acid substitution can lead to altered physiochemical properties of the tissue. End products or side chain modifications from biochemical modifications greatly compromise the mechanical function of the ECM, which have important physiological consequences. These biochemical reactions also include altered environmental niches for cells, which affect their health and development, and may lead to progressively increasing damage.

Great advances in high field solid state NMR have allowed for studies of a variety of proteins and peptides. Studies of elastin by these methods still remain a challenge due to its large size and highly cross-linked nature. Dynamic nuclear polarization, which provides improvement in signal enhancement, presently requires freezing samples to approximately 100K. However, at temperatures as low as only 273K the dynamics of elastin appear greatly reduced. Consequently, ^{13}C spectra of elastin appear broad and featureless, similar to lyophilized samples providing little structural and dynamical information (Perry et al. 2002). Thus, without domain and site-specific labels, molecular dynamics simulations will likely remain a useful tool for obtaining further thermodynamical, structural, and dynamical information of this protein. These methods, when combined in tandem with NMR, may be used to pick apart complexity of elastin and its interaction with a solvent. PFM has been applied to study electromechanical coupling in a variety of biological tissues and molecules, though a number of challenges need to be overcome before its potential application can be fully realized. Most importantly is that PFM can be performed under physiologically

relevant conditions, and differentiate electromechanical responses from various microscopic origins. Nevertheless, PFM could provide an alternative and highly sensitive method for investigating structure and property changes during early development of diseases. By correlating electromechanical coupling of tissues with various disease conditions, much can be learned, though this would require close collaborations across disciplines including engineering, physics, and medicine.

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Abbreviations

AAA	abdominal aortic aneurysm
AFM	atomic force microscopy
APoE	apolipoprotein E
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
ECM	extracellular matrix
MD	molecular dynamics
PFM	piezoelectric force microscopy
NMR	nuclear magnetic resonance
TEM	transmission electron microscopy

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Figure 1.

a) Histology image of porcine aorta, elastin-black, collagen-yellow, cells-red Reprint from (Zou and Zhang, 2011); b) TEM image reveals elastic lamina (EL) with adjacent collagen fibers (Coll) and smooth muscle cells (vSMC) Reprint from (Wang et al., 2015); and c) Multi-photon image ($350\mu\text{m} \times 350\mu\text{m}$) showing elastin (magenta) and collagen (green) fiber networks in the medial layer of the arterial wall.

Figure 2.

Multi-photon images of collagen in the adventitial layer (top), collagen (middle) and elastin (bottom) in the medial layer of a porcine thoracic aorta during equal biaxial strain (from 0 to 40%). Images are $110 \times 110 \mu\text{m}$. Reprint from (Chow et al., 2014).

Figure 3.

Representative Cauchy stress vs. Green strain curves showing the progression of the mechanical properties of arteries through four major stages due to elastin degradation after treating with elastase for 0, 6, 12, 26, 48, and 96 hours: initial-softening (IS), elastomer-like (EL), extensible-but-stiff (ES), and collagen-scaffold (CS) behavior. Note only the longitudinal direction is shown. Reprint from (Chow et al., 2013).

Figure 4. Stress vs. Strain in the circumferential direction. After incubated in glucose for up to 4 weeks, elastin shows an increase in stiffness as well as hysteresis. Reprint from (Wang et al., 2015).

Figure 5.

Piezoelectric and ferroelectric effects of elastin; (a) PFM mapping; (b) PFM phase-voltage hysteresis loop; and (c) PFM amplitude-voltage butterfly loop. Reprint from (Liu et al., 2013).

Figure 6.

Suppression of ferroelectricity in elastin by glucose treatment. (a) 3D topography mapping and switching spectroscopy PFM mappings of (b) remnant amplitude and (c) nucleation bias, where points with no switching characteristics are marked by blue; (d) phase-voltage loops and (e) amplitude-voltage loops at four representative points, showing that switching is suppressed for points within the blue area, but is observed for points outside of it. (f) Comparison of percentages of points showing no switching characteristics in the control and glucose-treated elastin ($n=5$) over a $90 \times 90 \mu\text{m}^2$ sample area, with 64 points probed in each sample. Reprint from (Liu et al., 2013).

Figure 7. Comparison of PFM amplitude of four different groups of mouse artery tissues; (a) PFM amplitude mappings corrected using damped driven harmonic oscillator model; (b) Histogram of corrected PFM amplitude distributions. Reprinted from (Liu et al., 2016).

Figure 8.

^1H - ^{13}C MAS cross polarization NMR spectra of porcine elastin in (top) water and (bottom) 2M glucose. Glucose exposed tissue exhibits a larger cross-polarization efficiency, pointing to more rigid structure. Additionally, evidence for an increased alpha helical signature in the alanine domains is noted, and a cross polarized glucose signal is observed. Peak assignments have been previously published elsewhere (Silverstein et al. 2015). Reprinted from (Silverstein et al. 2015).

Figure 9. Ramachandran angles for an alanine pentamer in (a) water and (b) 2M glucose. Note that for AAAAA and is a change or increase from beta-spiral to alpha-helical, consistent with the experiment NMR results. The color bar on the right indicates population, with the entire Ramachandran space normalized to unity. Reprinted from (Silverstein et al. 2015).

The analysis of variance (ANOVA) of peak value and mean value of corrected PFM amplitude (A_m) and resonant frequency (F_r) in four different groups of samples. Reprint from (Silverstein et al. 2015).

Table 1

	1 (n = 12)	2 (n = 12)	3 (n = 12)	4 (n = 12)	F value	p value
Peak value (A_m)	4.73±0.38 ^{*#}	7.44±0.27 ^{,\$@}	12.80±0.74 [^]	19.48±2.71 ^{&}	250.27	< 0.001
Peak value (F_r)	319.19±4.33 [#]	316.81 ± 3.85 [@]	314.73 ± 4.47 [^]	325.20 ± 4.17 ^{&}	13.86	< 0.001
Average value (A_m)	4.67 ± 0.49 ^{*#}	7.92 ± 0.29 ^{,\$@}	13.00 ± 0.85 [^]	19.42 ± 2.31 ^{&}	310.08	< 0.001
Average value (F_r)	319.37 ± 5.21	317.20 ± 3.98 [@]	315.84 ± 6.32 [^]	323.52 ± 4.50	5.25	0.003

Note:

- ^{*} p < 0.05 for 1 vs 2;
- [#] p < 0.05 for 1 vs 3;
- [&] p < 0.05 for 1 vs 4;
- ^{\$} p < 0.05 for 2 vs 3;
- [@] p < 0.05 for 2 vs 4;
- [^] p < 0.05 for 3 vs 4.

Table 2

Example correlation times measured in glucose treated elastin point to slower ^1H - ^{13}C fluctuations (larger τ_c) in comparison to the control sample (water only). Reprinted from (Silverstein et al. 2015).

Assignment	$\tau_c \times 10^{-6}$ [s]
Untreated Sample	
C β -Val, C γ -Val	4.55 ± 0.87
C β -Val, C β -Pro	1.99 ± 0.26
C α -Gly, C β -Leu	2.26 ± 0.53
Glucose Treated Sample	
C β -Val, C γ -Val	7.63 ± 1.69
C β -Val, C β -Pro	6.18 ± 1.09
C α -Gly, C β -Leu	6.10 ± 1.46

Table 3

Summary of the total retractive force (f_{tot}) and the ratio of the energetic term (f_e) to the total retractive force for the peptide [VPGVG]₅ in water and in 2M glucose (Silverstein). Results for the pentamer AAAAA are also presented, either in water or in 2M glucose. The root mean square fluctuations of the C α carbon are shown for the peptides in water and in 2M glucose; for VPGVG a marked decrease in the RMSF is observed when the peptide is in glucose solution in comparison to water only. Reprinted from (Silverstein et al. 2015).

T=310 K	f_{tot} [kJ/(mol Å)]	f_e/f_{tot}	RMSF [nm] (unstrained state)
[VPGVG] ₅			
Water	8.0	-0.02	0.50 ± 0.21
2M glucose	10.0	0.37	0.05 ± 0.01
AAAAA			
Water	29.3	0.23	0.09 ± 0.02
2M glucose	17.7	0.20	0.07 ± 0.02