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The role of structure in the nonlinear mechanics of cross-linked semiflexible polymer networks

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The microstructural basis of the characteristic nonlinear mechanics of biopolymer networks remains unclear. We present a 3D network model of realistic, cross-linked semiflexible fibers to study strain-stiffening and the effect of fiber volume-occupancy. We identify two structural parameters, namely, network connectivity and fiber entanglements, that fully govern the nonlinear response from small to large strains. The results also reveal distinct deformation mechanisms at different length scales and, in particular, the contributions of heterogeneity at short length scales. © 2012 American Institute of Physics. [doi:10.1063/1.3682779]

INTRODUCTION

The mechanical versatility of biological tissues and cells plays a central role in various physiological and biological functions. The materials providing intra- and extra-cellular mechanical support for the cells are defined by cross-linked networks of semiflexible fibers. Inside the cell, a cytoskeletal network of intracellular proteins consisting of actin filaments, microtubules, intermediate filaments, and other proteins stabilizes cell structure, transmits environmental cues, both mechanical and chemical, and modulates numerous cell functions, such as cell morphology, division, motility, and even apoptosis.1 Outside the cell, a network of proteins, with collagen as the primary backbone, forms the extracellular matrix, which can mediate mechanical signals to and from the cell.2 One hallmark of these networks, which is the focus of this paper, is their ability to stiffen at increasing strain,3 thereby enhancing tissue integrity and producing the characteristic nonlinear stress-strain curve observed in soft tissues.4 Understanding the link between the structure and the viscoelasticity of both natural and synthetic networks is important not only in cellular studies (e.g., cytoskeletal mechanics) but also for the design of biocompatible materials and pharmaceutical gels.

To obtain a physical understanding of the relation between network microstructure and the viscoelastic response, in vitro reconstituted networks have been useful as a minimal experimental model, with actin as the most studied biopolymer. Rheological measurements on reconstituted actin networks have been influential in providing an understanding of the mechanisms underlying the frequency-dependent behavior of living cells, termed power-law rheology.5 The elasticity of solutions of actin filaments at small strains have been successfully understood in terms of the confined entropic excitation and entanglement of the filaments,6 while the nonlinear stiffening behavior in the cross-linked networks have been variously interpreted to arise from the properties of the single filaments,7 mechanical prestress in the network,7 properties of the cross-linkers,8 and active rearrangement by motor proteins.9, 10 The complexity and the interconnectedness of the relevant physical features, which are not always resolvable experimentally, are partly responsible for our incomplete understanding of the origin of the nonlinear behavior.11 For this reason, concerted theoretical and computational studies have been invaluable in providing insights on the structure-property relation of semiflexible networks.12

Theoretical studies have attempted to trace the nonlinear bulk elasticity to the nonlinear force-extension relation of single filaments by assuming affine network deformation.3, 13 In this case, entropy determines the network elasticity at small strains, while enthalpy plays an increasingly bigger role at larger strains. Enthalpic effects have also been proposed as sources of nonlinear bond elasticity, which in turn causes stiffening.14 This statistical mechanical model is able to link polymer dynamics and the concept of prestress, which has been hypothesized to play an important role in cell mechanics.15 Another model that attempts to reconcile the observed power-law rheology and prestress is the glassy worm-like chain model, wherein sticky interactions between biopolymers allow exponential stretching of the relaxation spectrum, corresponding to a rough free energy landscape.16 On the other hand, it has been argued that an alternative mechanism involving network rearrangement and a transition from bending- to stretch-dominated deformation can cause nonlinearity even without the nonlinear force-extension relation of single chains.17–19 Further, numerical studies on two-dimensional (2D) (Refs. 19–22) and, more recently, three-dimensional (3D) (Ref. 17) networks show evidence of non-affine behavior. In particular, the degree of network affinity has been shown to be a function of strain17, 19 as well as characteristic length scales of the network, such as the average distance between cross-links $l_c$, fiber length $L$ and persistence length $l_p$.21

A key aspect that has not received sufficient attention is the length scale of material homogeneity. At
macroscopic level, where homogeneity may not be too great an oversimplification, the network elasticity has been explained and predicted theoretically\textsuperscript{3, 13} as well as validated experimentally\textsuperscript{23, 24} typically in terms of polymer concentration $c_m$ and cross-linker concentration $c_{cl}$. However, biological materials are invariably heterogeneous at small scales (e.g., \text~\mu m), both \textit{in vivo}\textsuperscript{25} and \textit{in vitro}\textsuperscript{26}. Not surprisingly, therefore, it has been recently realized that specific network architecture, such as number of filaments incident to a branch point\textsuperscript{27} and number of cross-links per filament,\textsuperscript{28} plays an important role in governing elasticity, especially in 3D networks. Such quantitative analyses of network microstructure are hampered experimentally by a lack of understanding of how networks self-assemble and cross-link. This results in uncertainties on how macroscopic quantities, such as $c_m$ and $c_{cl}$, translate to the actual network architecture at short length scales.\textsuperscript{29} Understanding network mechanics and architecture at such small scale is extremely important for studies of biological cells, not only to give insights into cytoskeletal mechanics, but also because cells interact with the surrounding extracellular environment through small (micro- to nano-) scale interactions.\textsuperscript{30}

In view of these, it is crucial to base the analysis on a realistic model of semiflexible network for which the microstructure can be quantitatively parameterized. Therefore, we develop discrete 3D semiflexible networks with tunable fiber dimensions and show that the nonlinear mechanics can be explained exclusively through the structural features of the network. Specifically, two parameters representing cross-link and fiber entanglement are shown to govern network stiffness at multiple strain levels and length scales relevant to the cell; thus, nonlinearity is linked directly to structural features. This is, to our knowledge, the first attempt to investigate the influence of 3D topological interaction between fibers in biophysical networks as explicitly modeled fiber steric hindrance. In addition, our approach can resolve the different deformation mechanisms that have been proposed to explain the experimental findings available in the literature.

METHODS

Network model

To model the fibers in the network, we modified the standard “shish-kebab model” typically used to represent coarse-grained polymer and worm-like chains.\textsuperscript{31} Each fiber was discretized as an array of beads of diameter $d$, with neighboring beads situated at an equilibrium distance of $d/2$, rather than $d$, from each other. We find that this helps in simulating fiber-fiber interaction and in enforcing excluded-volume effect stably, especially at large strains. The total number of beads per fiber was therefore determined by the initial fiber contour length, $L$, as $2L/d - 1$. The beads were connected by a harmonic stretching potential

$$U_s = \frac{1}{2} k_i (r - d/2)^2,$$

where $r$ is the distance between beads, and the fiber semiflexibility was implemented using a harmonic bending potential

$$U_b = \frac{1}{2} k_b (\theta - \pi)^2,$$

where $\theta$ is the angle formed between neighboring bonds. The respective stretching constant $k_i$ and bending constant $k_b$ can be directly related to experimentally accessible quantities $I_p$ and the fiber Young’s modulus $E$, as we have independently tested. The harmonic functional form of $U_s$ and $U_b$ is deliberately chosen not only because of its simplicity but also to make the results directly comparable to previous studies.\textsuperscript{17, 19, 21}

We also model two types of interactions between different fibers: entanglement and cross-link-mediated. The physical entanglement between non-penetrable fibers was modeled with a one-sided repulsive potential

$$U_i = \begin{cases} \frac{1}{2} k_i (d - r)^2, & r \leq d \\ 0, & r > d \end{cases},$$

where $r$ is the distance between non-neighboring beads. The interaction constant $k_i$ determines the “softness” or the degree of penetrability of the fibers. Although the functional form of $U_i$ can in principle be extracted from experimental data (e.g., from fiber indentation studies), the choice of the function is unlikely to affect the overall network response at physiological fiber volume fractions. Here, we employ $U_i$ simply to prevent fiber-fiber penetration. Cross-linking between fibers was represented by a harmonic cross-link potential

$$U_{cl} = \frac{1}{2} k_{cl} (r - r_0)^2.$$

The cross-link length $r_0$ and compliance $k_{cl}$ can influence network nonlinearity\textsuperscript{7, 8, 23, 32, 33} and deserve a full exploration on their own. Here, we generically set $k_{cl} \equiv k_i$, and $r_0 \equiv d$ to simulate permanent, freely rotating, chemically strong cross-links.

We summarize the fiber model and interaction in Figure 1. The key parameters that relate to typical experimental values and which are useful in repeating the simulations can be found in Table I. The fiber model employed here mimics cylindrical topology while maintaining short-scale bending flexibility, without excessive computational burden. Moreover, the resulting network allows fairly smooth 3D steric entanglement, which cannot be simulated in 2D simulations.

FIG. 1. Summary of fiber model and interactions.
Network generation and deformation

The networks were generated by initially placing $N$ straight fibers with random position and orientation in a periodic cubic cell of size $W \times W \times W$, with $N$ determined from the given polymer concentration $c_m$ or volume fraction $\Phi$. To study the short length-scale behavior of the network directly, as we shall discuss in more detail, we keep the cell size $W$ not larger than $2L$. A snapshot of this initial state is shown in Figure 2(a). Fibers that traverse the top and bottom walls—walls parallel to the shear plane—were shifted down or up, respectively, to make the networks simulate experimental conditions. Cross-links were assigned when two beads from different fibers were within a threshold distance $\delta_{cl}$, as illustrated in Figure 2(b). To avoid double counting and keep the simulation stable, when there was more than one bead within $\delta_{cl}$ from another bead, only the pair with the shortest distance was assigned as cross-links. This procedure has an advantage over previous numerical studies,\cite{17,19,21} as cross-link density can be varied independently of $c_m$ by varying $\delta_{cl}$. Before any mechanical test, these highly stressed networks were energy-minimized based on the total potential $U = U_c + U_b + U_{cl} + U_i$, allowing relaxation of the fibers and entanglement points. While there may be some amount of mechanical pre-stress that remains even after this relaxation step, we expect the effect to be completely random and comparable to that in standard mechanical experiments.\cite{7} The resulting network architecture, as shown in Figure 2(c), mimics self-assembled cross-linked biopolymer gels formed in vitro,\cite{24} which have been used to explain cell mechanics.\cite{34}

To deform the networks realistically, we defined top and bottom walls beads as the beads located within $\delta_w$ from the top and bottom walls, respectively. During the network straining, the bottom wall beads were fixed and the top wall beads were moved according to the imposed shear strain $\gamma$. Only one bead per fiber was allowed to be assigned as wall beads. The force needed to keep the wall beads in place for each strain level tested was sampled using Brownian Dynamics at 300 K, hence taking entropy into account and was then used to calculate the network stress $\tau$. Figure 2(d) shows a snapshot of the network topology in Figure 2(c) under a shear strain of $\gamma = 0.5$, and a general comparison clearly shows that a global network rearrangement has taken place through fiber reorientations and translocation, consistent with previous 2D as well as 3D studies.\cite{17,19}

RESULTS

We studied the nonlinear stiffening of the networks by incrementally imposing the shear strain $\gamma$ up to $\sim$100%. Shear stress $\tau$ was obtained from the force needed to impose a given strain $\gamma$. Figure 3 shows the complete stress-strain response for typical networks with $\Phi = 5.6\%$, 7.4\%, and 9.3\%. At small $\gamma$, the network responds linearly, with stiffness comparable to values measured for biopolymer networks.\cite{3,24} As $\gamma$ increases, the stiffness gradually increases, introducing nonlinearity in the response. In general, the overall response can be characterized by the small-strain stiffness $G_0 = \left[\frac{d\tau}{d\gamma}\right]_{\gamma \rightarrow 0}$, the critical strain $\gamma_c$, corresponding to the onset of nonlinearity, and the instantaneous large-strain modulus $G_L = \left[\frac{d\tau}{d\gamma}\right]_{\gamma = 3\gamma_c}$, as indicated in Figure 3. In practice, $\gamma_c$ was determined from the first data point that brought the correlation coefficient ($R^2$) in the linear fitting for $G_0$ lower than 0.99. The choice of $\gamma = 3\gamma_c$ in the calculation of $G_L$ is arbitrary but guarantees that $G_L$ is obtained well beyond the onset of stiffening. The stress-strain response is observably highly sensitive to variations in the network physical properties, e.g., $\Phi$ and cross-link density (even for fixed $\Phi$). In the following, we demonstrate how these variations can be described in terms of characteristic network structure parameters.
Parameterization of network structural heterogeneities

The role of network structure on the elasticity has been studied extensively, both theoretically and experimentally, especially at small strains. In particular, the small-strain stiffness \( G_0 \) is typically reported to vary as \( G_0 \sim c_m^x R^y \), where \( R = c_{cl}/c_m \) and the exponents \( x \) and \( y \) vary with biopolymer type as well as properties and strength of cross-linkers.\(^{13,18,24,33,35}\) While \( c_m \) and \( R \) are convenient to measure experimentally and reflect the overall properties of the network, these are macroscopic quantities with no direct link to the actual network architecture at different length scales. This is especially true in biological or biomimetic networks, which are notoriously heterogeneous, as a result of complex physicochemical self-assembly processes.\(^{25,26,29}\)

Without proper accounting for the exact mechanism of biopolymer self-assembly and cross-linking, these quantities can in fact provide misleading information on the local network microstructure, which is relevant at small length scales. A central message of this paper is that one can obtain better understanding on the roles of heterogeneity and length scales in the network mechanics through accurate parameterization of network structure, which we now describe.

To describe the actual network structure at short scales more faithfully, we directly quantify the structural features of the self-assembled networks in terms of two parameters: the number of entanglements per fiber, \( R_e \), and the number of cross-links per fiber, \( R_{cl} \). Here, \( R_{cl} \) can be adjusted arbitrarily through \( \delta_{cl} \) and \( \Phi \). In essence, the main difference between the experimental parameters, \( c_m \) and \( R \), and our structural parameters, \( R_e \) and \( R_{cl} \), is that the former are “input” parameters which are sometimes incorrectly assumed to unambiguously translate to the assembled network, while the latter are “output” parameters that directly quantify the structural result of the assembled network. We expect the two sets of parameters to converge as the networks become more homogeneous.

Figure 4 shows the relation between the actual \( R_{cl} \) obtained from network generation and the given values of \( \delta_{cl} \) and \( \Phi \), which is proportional to \( c_m \). The variation of \( R_{cl} \), as indicated by the error bars in Figure 4, reflects the variety of structural features that can result at small length scales for a given experimental variable such as \( c_m \). In addition, it underlines the effects and importance of accounting for local heterogeneity often encountered in biopolymer networks.\(^{22,36}\)

This realistic sample-to-sample variation makes it possible, for example, for a network of a lower \( c_m \) to have larger degree of connectivity than one with higher \( c_m \). The fact that, on average, the network structural parameter \( R_{cl} \) can be finely tuned by the two physical input parameters, \( \delta_{cl} \) and \( \Phi \), as seen in the inset of Figure 4, can thus be understood in analogy to the law of large numbers: as the number of network (spatial) sampling increases, the average of the parameters grows closer to the values expected for increasingly macroscopic, homogeneous networks. The number of averaging needed to bring the parameters close to the average value would in principle reflect the degree of heterogeneity of the network assembly.

Length-scale-dependent network mechanics at small strains

We start the analysis of network mechanics by looking at the network behavior at small \( \gamma \). We find that all \( G_0 \)'s from networks generated with different \( \Phi \) and \( \delta_{cl} \) fall into a master relation \( G_0 \sim R_{cl}^{0.8} \), as shown in Figure 5(a) (although the correlation coefficient \( R^2 \) is only 0.59). Again, note that here we simply quantify the resulting network structure through \( R_{cl} \) without distinguishing the cause, which can jointly arise from fiber and cross-link densities. This apparent loss of information, however, is recompensed by an intimate description of network structural properties that can account for the exact effects of biopolymer self-assembly and cross-linking, in contrast to previous models.\(^{13,23,24}\) Moreover, our finding not only confirms the predicted role of macro-scale network connectivity\(^{13}\) but also hints at a more universal mechanism of network deformation based principally on structural properties, which calls for deeper investigations on the nontrivial relation between the macroscopic inputs \( c_m \) and \( R \) and the actual network structure, as quantified through \( R_{cl} \) here.

The short-scale interaction between network fibers is characterized by entanglements, whose effect is subtler and weaker than that of cross-links.\(^{13}\) To study this local steric interaction more closely, we also quantify the number of en-
Nonlinear strain-dependent network mechanics

We now shift our focus to the network nonlinear behavior. The strain required to trigger stiffening, $\gamma_c$, is known to indicate the transition in the underlying mechanics, e.g., bending- to stretch-dominated.\textsuperscript{13,22,37,38} Our results on the role of network structure on $\gamma_c$, shown in (a) and (b) against $R_{cl}$, respectively, exhibit scaling relations with $R_{cl}$ at all volume fractions tested ($\wedge$, $\Phi = 9.3\%$; $\blacksquare$, $\Phi = 7.4\%$; $\bullet$, $\Phi = 5.6\%$), demonstrating the importance of network structure parameters even beyond the linear elastic regime shown in Figure 5.

FIG. 5. Influence of network structure on the network response at small strain. Small-strain stiffness $G_0$ is plotted in (a) against $R_{cl}$ and in (b) against $R_c$. The data are obtained from networks with fixed fiber dimensions but various $\Phi$ ($\wedge$, $\Phi = 9.3\%$; $\blacksquare$, $\Phi = 7.4\%$; $\bullet$, $\Phi = 5.6\%$) and cross-link densities, $0.5 < \delta/d < 1.5$, resulting in networks with varying connectivity. The relation between the averaged macroscopic quantity $R_{cl}$ and the averaged microscopic quantity $R_c$ is shown in (c). The dashed line is a guide for the eye.

FIG. 6. Influence of network structure on the network response at intermediate and large strains. Both the critical strain $\gamma_c$ and the large-strain stiffness $G_L$ shown in (a) and (b) respectively, exhibit scaling relations with $R_{cl}$ at all volume fractions tested ($\wedge$, $\Phi = 9.3\%$; $\blacksquare$, $\Phi = 7.4\%$; $\bullet$, $\Phi = 5.6\%$), demonstrating the importance of network structure parameters even beyond the linear elastic regime shown in Figure 5.
Network deformation mechanism

One way to quantify the importance of heterogeneity in the deformation mechanism is by analyzing the extents of affinity and fiber rearrangements during network deformation. Networks with higher degree of homogeneity are likely to distribute stress more uniformly, leading to more affine deformation. The affinity (or nonaffinity) of deformation is typically measured by looking at the difference in length, angle, or vector position between the observed deformation and that predicted for a purely affine deformation, as illustrated in Figure 7, and can be quantified in a number of ways.40 Here, we use the dimensionless nonaffinity parameter $A$, which is similarly used in previous 3D network study,17 as defined by

$$A = \left( \frac{\|\delta u_{cl} - \delta u_{cl}^{aff}\|^2}{\|\delta u_{cl}^{aff}\|^2} \right),$$

where $\delta u_{cl}$ denotes the cumulative nonaffine rearrangement that has occurred. Figure 8 shows that the amount of rearrangement increases continually with $\gamma$, with a significant portion occurring even before the network stiffening, as signified by the steeper slope $dV/d\gamma$ at $\gamma < \gamma_c$. These trends of $A$ and $V$ with $\gamma$ therefore provide independent supports for our earlier remark that network heterogeneity plays increasingly smaller role at larger $\gamma$.

DISCUSSION

One of the problems in extrapolating experimental results and interpretations to small, fiber- or cell-level scales is the difficulty in determining how experimental parameters such as $c_m$ and $R$ translate to these length scales. In many cases, (both experimental as well as theoretical), spatial homogeneity is assumed to be sufficient to develop a first-order understanding of the physics and mechanics of the network behavior. However, extrapolation to physiological, or even in vitro, systems is inevitably complicated by different network compositions, microstructural heterogeneity, self-assembly and cross-linking machineries, all of which are expected to alter the network’s structural and mechanical properties. In this work, we attempt to circumvent these difficulties by directly quantifying the structural parameters of the network in terms of $R_{cl}$ and $R$, with all the complexity and heterogeneity that result from the biopolymer self-assembly, rather than simply taking the known experimental parameters $c_m$ and $R$ as macroscopic representations for the actual microscopic structural determinants of mechanics. From our results, we find that these structural parameters can in fact help one understand the interplay of the network connectivity and topology at different length scales relevant to the cell. Importantly, we note that these parameters, which can be obtained straightforwardly in computer simulations, are also accessible experimentally, in particular, advances in today’s imaging modalities, such as confocal fluorescence microscopy and second harmonic generation imaging, may prove useful similarly to predict local network responses from 3D structural images of the assembled network.28,38,41

The contributions from entanglement effects and cross-links to the mechanics at small strains have been studied theoretically and experimentally previously in the context of actin networks.42,43 It was found that, below a critical value of $R$, the concentration-dependent $G_0$ agrees with the theoretical prediction for entangled actin solution entanglements effects, and this has been described in terms of the Odijk length $l_o$ and the resulting suppression of thermal excitation.45 At the
other end, above the critical value of $R$, $G_0$ is strongly dependent on $R$, indicating the role of cross-links in the network mechanics. A comparison between bulk rheology and micro rheological measurements reveals significant heterogeneity within biopolymer networks and an increasing degree of homogeneity with cross-linker concentration. Moreover, when spatially averaged, micro rheology leads to results consistent with those from bulk rheology, as we also find. Taken together, these studies therefore provide experimental support for our hypothesis that the relative roles of fiber entanglement and cross-links need to be considered in the context of network heterogeneity and measurement length scale.

Furthermore, our results show that the contributions of entanglement effects and cross-links on the nonlinear behavior of the network are in fact also functions of strain: the larger the strain, the smaller the contribution of entanglement and the larger the contribution of cross-links. The diminishing entanglement effect is accompanied by the increase of network affinity, which reflects the degree of homogeneity in the network response. In effect, we have directly shown that the applicability of the entanglement theory (and theoretical predictions on cross-linked semiflexible polymers) to network mechanics is highly linked to the local topological features of the network, which vary not only with the macroscopic cross-linker concentration, but also with measurement length scales, level of strain, and degree of heterogeneity. As a result, fiber entanglement must also be taken into account in the low-strain limit of the cross-linked polymer network, where cross-links are not yet actively involved in the network deformation, especially in the small length scales relevant to the cell.

The variety of biopolymer self-assembly and cross-linking machineries, among other factors, contributes significantly to the microstructure of the resulting networks. For example, the interplay between the monomer size and the cross-linker properties may determine the fiber dispersity and propensity to bundling, which in turn will affect the strain transmission mechanism and the overall mechanical behavior of the network. By choosing the effective potential energies described in the Methods section and variable values listed in Table 1, we have in the present study focused our attention to the mechanics of a subset of biopolymer systems. However, we expect our approach to be robust and applicable to understanding the structural origin of the mechanics of a wide range of biophysical networks. In addition, our simulation methodology allows similar studies to be done at different frequencies. Thus, the network model employed here can potentially provide microstructural insights into the physical basis of the experimentally observed power law rheology of biopolymer networks at different length scales. Nevertheless, there remain many important challenges before what is known about the mechanics of biopolymer networks can be fully utilized in understanding cellular mechanics, as discussed in excellent recent reviews. For example, cells have been reported to fluidize, instead of elastically stiffen, in response to large stresses. Importantly, the extent of fluidization depends on both the cytoskeletal prestresses and the concurrent stiffening behavior. Such a phenomenon might be caused by specific intra- and inter-chain interactions that call for more realistic modeling of the entanglement and cross-link properties.

**CONCLUSIONS**

In summary, we demonstrate that (i) structural properties, namely, network connectivity and physical entanglements, lie at the heart of the nonlinear mechanics of 3D networks, (ii) the contributions from these two parameters define the dominant deformation mechanism, e.g., affine vs. nonaffine, and (iii) the crossover between the two is governed by both length scale of observation and strain level. For $\gamma < \gamma_c$, the overall deformation is dominated by short-scale fiber mechanics, and the network stiffness is governed by steric interaction at short length scales and by cross-links at larger length scales. But once the network is sufficiently strained, reorganization causes the effects of local heterogeneity to diminish and be replaced by a more homogeneous response. Biological networks may thus take advantage of the heterogeneity at small \( \gamma \) to accommodate various functions, while retaining the ability to accurately control large-strain responses through active cross-linkers for the benefit of network integrity.

The insights obtained on the distinct deformation mechanisms at different length scales provided by the model employed here underscore the role of 3D steric interactions between fibers and may not be accessible through previous models. Our simple model also allows easy adaptation fashioned to diverse lines of exploration. Future work can be aimed at elucidating the individual roles of fiber properties (e.g., $d$, $L$, $l_p$, and $E$) and cross-linker properties (e.g., $k_{cl}$ and $r_0$), which will enable more quantitative comparison to other experimental and theoretical works. These properties, or the distribution thereof, are likely to affect the structural network response by varying the length-scale difference between the heterogeneous and the homogeneous scales.

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involved. This is reflected in the limitations imposed by the size of the independent variable. However, this is not always feasible in biological systems, in view of the restricted ranges of the parameters involved. This is reflected in the limitations imposed by the size of the parameter system we consider here.


