Interplay between viscoelasticity and stiffness as essential properties in mechanobiology

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Abstract

Over the last ten years, there has been a significant surge in mechanobiological research aimed at elucidating how substrate stiffness alters cell biology and the stiffness of multicellular systems. Although the concept of "stiffness," is employed to describe the material characteristics of both cells and their substrates, it lacks a precise definition within the realms of soft matter physics and rheology. Generally, stiffness can be understood as the degree to which a material resists deformation when subjected to a mechanical force. It is well established that both multicellular systems and substrate matrices exhibit viscoelastic behaviour. The stiffness of these viscoelastic systems is not constant; rather, it is influenced by the interplay between energy storage, which tends to increase stiffness, and energy dissipation, which tends to reduce it. These processes can occur simultaneously or at different rates. The traction forces exerted by cells can induce alterations in the substrate matrix, thereby modifying its stiffness, which in turn affects the efficiency of cell migration and the overall stiffness of a multicellular system. The mechanisms through which cells detect variations in the viscoelastic properties of their surroundings are still being explored, and they are influenced by the rates and magnitudes of energy storage and dissipation. This discussion of stiffness is contextualized through the examination of collective migration in epithelial and mesenchymal monolayers on collagen I matrices, drawing on both experimental findings and rheological constitutive models.

Key words: cell migration, cell contractility, adhesion contacts, stress relaxation time, energy storage and dissipation

Glossary of terms

Cell jamming state transition: the cell transition from the active (contractile) to the passive (non-contractile) state driven by the accumulation of compressive stress. This transition enhances the packing density of the cells and impacts the viscoelastic behaviour and surface characteristics of multicellular systems.
Energy dissipation: the quantity of energy that is lost as a result of structural changes within a system when subjected to external forces.
Energy storage: the quantity of energy retained within a system as a result of applied forces.
Constitutive models: stress-strain relationships.
Mechanical stress: a physical parameter that indicates both the intensity and orientation of forces applied per unit area, which result in deformation.
Normal stress: the stress that acts perpendicular to a surface. When this stress is positive, indicating an outward direction relative to the surface, it is classified as tensional stress. Conversely, if the stress is negative, acting in the opposite direction, it is categorized as compressive stress.
Residual stress: stress that remains in a system in the absence of external forces. Residual stress may be classified as dissipative (viscous) or elastic in nature.
Shear strain: deformation of a system in response to mechanical stress applied tangentially.
Shear stress: the stress that operates in a plane parallel to the cross section of a system.
Strain rate: the change in strain per unit of time.
Stress relaxation: the phenomenon where the stress within a system gradually decreases over time from an initial value to a residual stress state, all while maintaining a constant strain.
Viscoelasticity: a characteristic of systems that display both viscous and elastic behaviour when subjected to forces. These forces may arise from external or internal sources. This property is associated with the system's capacity to relax when experiencing strain or stress.
Volumetric strain: a type of strain that results in a variation of volume. In the context of linear analysis, particularly when dealing with small strains, it is calculated as the trace of the strain tensor.

1. Introduction

Numerous studies in mechanobiology have focused on characteristics such as the stiffness or rigidity of substrates and cells. Mechanical signalling refers to a mechanism in which a mechanical stress applied to biomolecules, initiates a signal [1-4). The mechanical stress caused by collective cell migration typically results in conformational changes in proteins or receptors, thereby revealing functional domains to the surrounding environment. The intricate nature of physical stimuli and the cellular responses they elicit is comparable to the complexity associated with chemical signalling. Although the term "stiffness" is commonly employed to describe the properties of materials in both cellular and substrate contexts, it lacks a precise and universally-recognized definition, especially for viscoelastic systems [1]. Generally, stiffness has been often quantified by Young's modulus. However, it is important to note that multicellular systems and substrate matrices exhibit viscoelastic behaviour, leading to an effective stiffness that is time-dependent.

Viscoelasticity is not a characterization of a system's state; rather, it pertains to the manner in which a system adjusts to forces being applied, either externally or internally. This adjustment occurs through a time-dependent mechanism involving the storage and dissipation of elastic energy as the structure of the system undergoes changes [5]. When the energy stored exceeds the energy dissipated, the system exhibits characteristics typical of a viscoelastic solid. Conversely, when energy dissipation surpasses energy storage, the system behaves as a viscoelastic liquid. The storage contributes to the stiffening of a soft-matter system, whereas energy dissipation leads to its softening. The primary features of viscoelastic liquids include: (i)the inability of strain to relax under constant stress conditions, (ii)the potential for strain rate to relax under certain conditions, and (iii) the possibility for stress to relax under constant strain rate conditions [5]. In contrast, viscoelastic solids exhibit markedly different behaviour. Their key characteristics are: (i)the ability of strain to relax under constant stress conditions and (ii) the capacity for stress to relax under constant strain conditions. When stress does relax, it tends to approach the (equilibrium) residual stress. In viscoelastic solids, the residual stress can be classified as: (i)elastic or (ii)a combination of both viscous and elastic components. Conversely, the residual stress in linear viscoelastic liquids is entirely dissipative, characterized solely by viscous stress. Within the same system, various scenarios of energy storage and dissipation can occur, contingent upon various loading conditions. Notable examples are (i) the response of a strongly-connected multicellular aggregate subjected to uni-axial compression between parallel plates[6,7] and (ii)cell aggregate micropipette aspiration[8]. The response of cell aggregates to uniaxial compression is indicative of linear viscoelastic solids. This observation aligns with the principle that stress can exhibit exponential relaxation under a constant strain, while strain may relax under constant or zero compressive stress[6]. The associated stress relaxation time spans several minutes and is linked to the remodelling of cell-cell adhesion contacts and alterations in cell morphology[6,9]. In contrast, strain relaxation occurs through collective cell migration over extended periods, typically hours [6]. Micropipette aspiration targets a localized area of the multicellular aggregate, leading to the disruption of cell-cell adhesion and prompting cell migration towards the micropipette resulting in significant energy dissipation. Under these conditions, both cell strain and strain rate are unable to relax, whereas stress can still undergo exponential relaxation under a constant strain rate[8].

The behaviour exhibited by multicellular aggregates aligns with that of linear viscoelastic liquids. However, this approach to assessing the viscoelastic properties of multicellular aggregates is not applicable as a measure of the viscoelastic behaviour of migrating cell collectives in the absence of externally applied forces. It is well established that cell migration leads to the generation of strain,

which evolves over hours. This strain subsequently induces mechanical stress. The classification of multicellular systems as viscoelastic solids or liquids is primarily influenced by the strength of cell-cell adhesion, or cell cohesion. Epithelial cells form strong E-cadherin-mediated adhesion contacts, allowing them to migrate as cohesive cell aggregates, which exhibit characteristics of viscoelastic solids[10]. Serra-Picamal et al.[11] and Notbohm et al.[12] examined the collective migration of Madin-Darby canine kidney type II(MDCK) cells at packing densities equal to or lower than that of a confluent state, highlighting that the residual stress generated is correlated with the associated strain. In this scenario, changes in strain and the generation of residual stress occur over hours, while stress relaxation occurs within minutes. Pajic-Lijakovic and Milivojevic[13] proposed the following scenario for the viscoelasticity of migrating epithelial collectives with cell packing density lower than or equal to the cell packing density in the confluent state. Collective cell migration induces a step-by-step increase in strain over hours. Every strain causes an increase in mechanical stress and its relaxation within many short-time relaxation cycles under constant strain per cycle. These successive stress relaxation cycles result in the generation of cell residual stress and its increase over hours. Cell mechanical stress, cell packing density, cell velocity, corresponding strain, and traction forces are inhomogeneously distributed within cell monolayers[14,15]. An increase in cell packing density, caused by the accumulation of mechanical stress, supresses cell stress relaxation and changes the rheological behaviour of epithelial systems. Otherwise, mesenchymal cells establish weak cell-cell adhesions and migrate in the form of streams [10,16]. The viscoelasticity of migrating collectives of mesenchymal cells corresponds to that of viscoelastic liquids [17].

The viscoelasticity and stiffness of cell monolayers, along with the effectiveness and persistence of collective cell migration, are strongly influenced by the viscoelastic properties and stiffness of the substrate matrices. Most substrate matrices, such as collagen networks, exhibit viscoelastic behaviour[18], with collagen networks serving as primary components of various soft tissues. Extensive research has focused on the properties of cell migration in relation to the stiffness of substrate matrices; however, the influence of the viscoelasticity of collagen networks on cellular responses remains poorly understood. Both monolayers and substrate matrices in direct contact undergo continuous multi-time changes, interacting with one another in complex cycles of cause and effect that influence the stiffness of both systems. The interconnection of these processes and their contributions to cell migration continue to be a subject of debate.

The stiffness of cells and of the substrate matrix are critical factors influencing cellular responses during development and disease. This stiffness is inherently linked to the viscoelastic properties of both the cells and the substrate with which they interact. In what follows, we aim to enhance our understanding of viscoelasticity in the context of energy storage and dissipation. To achieve this, we will examine established constitutive models for migrating epithelial and mesenchymal monolayers on a collagen I matrix, analysing their relevance to stiffness. Our objectives are to: (i)identify the primary physical parameters that contribute to the stiffening of cell monolayers and substrate matrices, (ii)explore how cell-generated tractions influence the formation of stiffness gradients within the matrix, (iii)assess the effects of matrix stiffening on the stiffness of monolayers, and (iv)evaluate how the rate of energy dissipation within the matrix affects the efficiency of cell migration.

2. Stiffness of multicellular systems: the impact of viscoelasticity

Collective cell migration generates strain. This strain leads in turn to the generation of mechanical stress. Both strain and stress play significant roles in energy storage and dissipation processes. The accumulation of energy causes the reinforcement of cell-cell and cell-matrix adhesions, which lead to

stiffening of contractile multicellular systems, whereas energy dissipation contributes to their softening [19-21]. The overall stiffness of the system is thus a manifestation of a competition between these two opposing effects. Stress relaxation, when it occurs, typically transpires over a time frame of minutes, while changes in strain and the generation of residual stress unfold over hours. As a result, the accumulation of elastic energy and energy dissipation within migrating cell collectives may occur simultaneously on the same time scale of hours, or on differing time scales, such as minutes and hours, depending on the selected constitutive model. The energy stored due to increased cell contractility, which enhances the strength of cell-cell and cell-matrix adhesion, as well as an increase in cell packing density, can be expressed quantitatively as: $W_s(r,\tau) = W_s\left(\tilde{\boldsymbol{\sigma}}_{\boldsymbol{el}}, \tilde{\boldsymbol{\varepsilon}}, \dot{\tilde{\boldsymbol{\varepsilon}}}\right)$, where $\tilde{\boldsymbol{\sigma}}_{\boldsymbol{el}}$ is the elastic part of stress, $\tilde{\boldsymbol{\varepsilon}}$ is the strain, the strain rate $\dot{\tilde{\boldsymbol{\varepsilon}}} = \frac{d\tilde{\boldsymbol{\varepsilon}}}{d\tau}$, and τ is the time scale in hours over which the cell migration occurs). The strain includes shear $\tilde{\boldsymbol{\varepsilon}}_{\boldsymbol{S}}(r,\tau)$ and volumetric $\tilde{\boldsymbol{\varepsilon}}_{\boldsymbol{V}}(r,\tau)$ contributions (where $\tilde{\boldsymbol{\varepsilon}}_{\boldsymbol{V}}(r,\tau) = (\overrightarrow{\boldsymbol{\nabla}} \cdot \vec{\boldsymbol{u}})\tilde{\boldsymbol{I}}$ and $\tilde{\boldsymbol{\varepsilon}}_{\boldsymbol{S}}(r,\tau) = \frac{1}{2}\left(\vec{\boldsymbol{\nabla}}\vec{\boldsymbol{u}} + \vec{\boldsymbol{\nabla}}\vec{\boldsymbol{u}}^{\boldsymbol{T}}\right)$, while $\vec{\boldsymbol{u}}$ is the displacement field, and $\tilde{\boldsymbol{I}}$ is the unit tensor). The energy dissipation caused by: (i)slow-down of cell migration, (ii)weakening of cell-cell adhesion contacts, and (iii)decrease in cell contractility, can be expressed as: $W_d(r,\tau) = W_d\left(\tilde{\boldsymbol{\sigma}}_{\boldsymbol{vis}}, \tilde{\boldsymbol{\varepsilon}}, \dot{\tilde{\boldsymbol{\varepsilon}}}\right)$ where $\tilde{\boldsymbol{\sigma}}_{\boldsymbol{vis}}$ is the viscous part of the stress). Our discussion will focus on two distinct types of migrating multicellular system: epithelial collectives and mesenchymal collectives. Migrating epithelial collectives, which exhibit strong intercellular connections, behave as viscoelastic solids. In contrast, mesenchymal cells, characterized by weaker N-cadherin mediated connections, migrate in a form of streams, behave as viscoelastic liquids[10,17,22].

The viscoelasticity of migrating epithelial monolayers on substrate matrices for cell packing densities lower than or equal to the cell packing density in the confluent state satisfies the conditions: (i) the generated stress correlates with the corresponding strain [11,12,23], and (ii) stress can relax under externally induced or internally generated strain conditions [6,9]. The ability of stress to relax under constant strain conditions points to viscoelastic solid behavior of epithelial monolayers. The exponential relaxation indicates a linear constitutive model. The suitable constitutive model, which satisfies all these conditions is the Zener model. The corresponding mechanism of cell migration is convective [22,24]. An increase in cell packing density, resulting from the accumulation of compressive stress, leads to a reduction in cell migration. In this scenario, the mechanism underlying cell migration shifts from a convective process to a diffusive one, with the average speed of cells decreasing by nearly two orders of magnitude. The relaxation of cellular stress is hindered in this densely packed environment. Given that diffusion operates as a linear mechanism, it becomes essential to introduce an alternative linear constitutive model for viscoelastic solids that adheres to the condition of non-relaxation of stress. The Kelvin-Voigt model has been suggested as a suitable candidate for this purpose [22]. An additional increase in cell packing density further inhibits cell migration, because it results in a transition to the jamming state, specifically a shift from a contractile to a non-contractile cell state [25]. Compressive stress levels in the range of several kPa can lead to cell jamming within three-dimensional multicellular systems [26]. Additionally, compressive stress values of a few hundred Pa, which occur during the collective migration of two-dimensional multicellular systems, are sufficient to initiate cell jamming [12]. In this context, cell migration is characterized by a non-linear sub-diffusion mechanism, which can be described using fractional derivatives. Fractional derivatives extend the concept of ordinary differentiation to non-integer (fractional) orders [28]. These derivatives are applied to describe anomalous diffusion (i.e., sub-diffusion and super-diffusion) and viscoelastic models. Pajic-Lijakovic and Milivojevic [22] have proposed a corresponding fractional constitutive model for cells in proximity to the jamming state.

The viscoelastic behavior of migrating mesenchymal collectives was characterized using the Maxwell model [8]. This model is defined by two key features: (i) the inability of strain to relax, and (ii) the

capacity for stress to relax under conditions of constant strain rate. The migration mechanism of mesenchymal collectives is also convective, with average cell speeds being comparable to, or slightly exceeding, those of migrating epithelial collectives at equivalent cell packing densities [27]. Further analysis necessitates delineation of the elastic and viscous components of the cell stress, which will inform the energy storage and dissipation mechanisms in the constitutive models proposed for both epithelial and mesenchymal cells during collective migration. The proposed models are shown in **Table 1**:

Table 1.

The storage and dissipation of energy in cell monolayers resulting from cell migration are influenced by the magnitude and nature of the strain produced. The collective movement of epithelial monolayers can lead to either uni-axial or bi-axial extension. Uni-axial extension is associated with sustained cell migration, whereas bi-axial extension is indicative of more chaotic cell movement. The occurrence of bi-axial extension results in an increase in the volume of the affected cell region and may create local gaps within the monolayer. In contrast, uni-axial extension induces transverse contraction strain, which is essential for maintaining the integrity of the multicellular structure. The extent of transverse contraction depends on the Poisson's ratio ν. A system's volume can: (i)increase for $\nu < 0.5$, (ii)stay constant for $\nu = 0.5$, or (iii)decrease for $\nu > 0.5$. Moisdon et al.[29] conducted measurements of the Poisson's ratio for MDCK and HeLa epithelial monolayers situated on a flat substrate, utilizing a frequency-mode approach across a frequency spectrum of $10^{-2} - 10^{2}$Hz. This frequency range is associated with structural alterations occurring over milliseconds to minutes. The findings revealed that the Poisson's ratio remains constant across frequencies, with a value of approximately ν ~ 0.77, signifying the compression of the monolayers.

The storage and dissipation of elastic energy within a migrating cell collective change the energetic state of a single cell on two time-scales, i.e., minutes and hours. The short-term change in the energy $\langle e_c \rangle$ after the increment of time Δt primarily depends on remodelling of the cell-cell and cell matrix adhesion contacts and can be expressed as:

$$\langle e_c \rangle(r, t + \Delta t, \tau) = \langle e_c \rangle(r, t, \tau) + \left(\frac{\partial \langle e_c \rangle_r}{\partial \rho_{CC}}\right)_{\rho_{CM}} \frac{d\rho_{CC}}{dt} + \left(\frac{\partial \langle e_c \rangle_r}{\partial \rho_{CM}}\right)_{\rho_{CC}} \frac{d\rho_{CM}}{dt} \qquad (1)$$

where ρ_{CC} and ρ_{CM} are the density of established cell-cell and cell-matrix adhesion contacts per single cell, respectively. While the densities $\rho_{CC}$and ρ_{CM}, change within minutes, the other relevant parameters such as: the number of contractile units N_{CU}, cell packing density n, and effective temperature T_{eff} change over hours. The cell packing density $n(r, \tau)$ is equal to $n(r, \tau) = \sum_i (r - r_i(\tau))$. The effective temperature is equal to $(k_B T_{eff})^{1/2} \sim \langle \|\vec{v}_c\| \rangle$[22,30], $\langle \|\vec{v}_c\| \rangle$ is the average cell speed, and k_B is Boltzmann's constant.

Cell speed is influenced by the density of cell packing, which can be associated with different transport mechanisms: a convective mechanism for $n(r, \tau) \leq n_{conf}$, a diffusion mechanism for $n_{conf} < n(r, \tau) < n_j$, and a sub-diffusion mechanism for $n(r, \tau) \to n_j$ (where n_c is the cell packing density in the confluent state and n_j is the cell packing density in the jamming state). Petitjean et al.[31] demonstrated that the MDCK monolayers reached the confluent (i.e., dense) state for a cell packing density of $n_{conf} \sim 2.5x10^5 \frac{\text{cells}}{\text{cm}^2}$ and a cell velocity of $\sim 0.14 \frac{\mu\text{m}}{\text{min}}$. The cell packing density n_j is an order of magnitude higher than n_{conf}[32]. Contractile units (CUs), which function as complexes that sense cellular rigidity, are characterized by a modular architecture measuring 2–3μm in length. These units congregate at the periphery of the cell upon initial interaction with a substrate matrix, preceding the formation of stress fibers and other components of the cytoskeleton[33]. Composed of myosin IIA,

actin filaments, tropomyosin 2.1(Tpm 2.1), α-actinin, and other cytoskeletal proteins, these units play a crucial role in regulating the strength of FAs between the cell and the extracellular matrix, which is influenced by the substrate's stiffness. On rigid surfaces, CUs promote the maturation of FAs, which are frequently associated with enhanced cellular proliferation[33]. In contrast, on softer substrates, the contractions of these units are transient, resulting in a swift disintegration of adhesions. Notbohm et al.[12] employed the concentration of phosphorylated myosin to elucidate aspects of cell contractility.

The long-term change in the energy after the increment of time $\Delta\tau$ depends on the cell packing density, the effective temperature and the number of contractile units. It can be expressed as:

$$\langle e_c\rangle(r, t_{eq}, \tau + \Delta\tau) = \langle e_c\rangle(r, t_{eq}, \tau) + \left(\frac{\partial\langle e_c\rangle_r}{\partial N_{CU}}\right)_{T_{eff},n} \frac{dN_{CU}}{d\tau} + \left(\frac{\partial\langle e_c\rangle_r}{\partial Teff}\right)_{n,N_{CU}} \frac{dT_{eff}}{d\tau} + \left(\frac{\partial\langle e_c\rangle_r}{\partial n}\right)_{T_{eff},N_{CU}} \frac{dn}{d\tau} \quad (2)$$

where t_{eq} is the equilibrium time after a short-time stress relaxation cycle.

The extension and compression of monolayers can be described by:

- The Zener model (**Table 1**) satisfy the conditions that: (i) $\langle e_c\rangle(r, t + \Delta t, \tau) = \langle e_c\rangle(r, t, \tau) - \Delta W_d$ and (ii) $\langle e_c\rangle(r, t_{eq}, \tau + \Delta\tau) = \langle e_c\rangle(r, t_{eq}, \tau) + \Delta W_s$,
- The Kelvin-Voigt model satisfy the conditions that: $\langle e_c\rangle(r, t_{eq}, \tau + \Delta\tau) = \langle e_c\rangle(r, t_{eq}, \tau) + \Delta W_s - \Delta W_d$,
- The Fractional model also satisfy the conditions that: $\langle e_c\rangle(r, t_{eq}, \tau + \Delta\tau) = \langle e_c\rangle(r, t_{eq}, \tau) + \Delta W_s - \Delta W_d$,
- The Maxwell model satisfy the conditions that: (i) $\langle e_c\rangle(r, t + \Delta t, \tau) = \langle e_c\rangle(r, t, \tau) + \Delta W_s$ and (ii) $\langle e_c\rangle(r, t_{eq}, \tau + \Delta\tau) = \langle e_c\rangle(r, t_{eq}, \tau) - \Delta W_d$.

A schematic representation of the various physical parameters which influence energy storage and dissipation, and their inter-relationships, is shown in **Figure 1**:

Figure 1.

The extension and compression of epithelial monolayers, caused by migration of epithelial collectives, were discussed in the context of change the stiffness in the context of proposed physical parameters. The result is presented in **Table 2**:

Table 2.

The accumulation of elastic energy results in an increase in the stiffness of a system, whereas energy dissipation leads to a reduction in stiffness. The stiffness is time-dependent and is a manifestation of the interplay between these two opposing processes, which may occur simultaneously or at different rates, depending on the strength of cell-cell and cell-matrix adhesions (focal adhesions), as well as the density of cell packing. Focal adhesions (FAs) are complexes composed of multiple proteins that include integrins, serving as mechanical connections between intracellular actin bundles and the extracellular matrix. Extension of monolayers leads to the reinforcement of cell-cell adhesion contacts [8], enhancement of cell contractility [34], weakening of FAs [35], an increase in cell speed [34], and a slight decrease in cell packing density. In this case, the reinforcement of cell-cell adhesion contacts and an increase in cell contractility, dominantly induces stiffening of epithelial monolayers [20]. Epithelial monolayers exhibit significant tensile strength, capable of enduring multiple times their original length prior to the onset of fracture [20].

An increase in cell packing density, caused by the system compression results in the stiffening of epithelial monolayers if and only if the cells maintain their contractile state and the integrity of cell-cell adhesion contacts[19]. When this transport mechanism is compromised due to weakened cell-cell adhesion, the contractility of individual cells may lead to single-cell stiffening; however, this effect does not extend to the stiffness of a multicellular system as a whole. It is in accordance with the fact that contractile cells exhibit significantly greater stiffness compared to their non-contractile counterparts. Research conducted by Schulze et al.[36] demonstrated that the Young's modulus of a contractile MDCK monolayer is approximately 33.0 ± 3.0 kPa, whereas the modulus for non-contractile cells is roughly half of that value.

Epithelial cells maintain their cell-cell adhesion contacts and contractility under lower and medium compression. In these cases, migrating epithelial collectives increase their stiffness[21]. However, high compressive stress intensifies contact inhibition of locomotion, leading to cell jamming. The contact inhibition of locomotion is a consequence of cell head-on interactions, leading to cell repolarisation and weakening of cell-cell and cell matrix adhesion contacts accompanied by suppression of cell contractility leading to softening of epithelial monolayer under jamming state[37,38]. Under jamming, epithelial monolayers become softer[19].

In contrast to epithelial cells, the migration of mesenchymal cells is more dissipative caused by weakening of cell-cell adhesion contacts[10]. The primary factor underlying the divergent behaviour of epithelial and mesenchymal cells, which are crucial for the transport of elastic energy throughout the monolayers is the strength of cell-cell adhesion contacts[24]. In this case, dissipative nature of cell migration is dominant, while energy storage, as well as the system stiffness decreases rapidly over minutes.

3. Stiffness of viscoelastic substrate matrix induced by cell tractions

The subcutaneous layer of skin is comprised of approximately 65% collagen, while tendons exhibit a collagen content of around 78%. We now explore the viscoelastic properties of collagen networks, particularly in relation to collective cell migration, and we examine how their viscoelastic characteristics influence the behaviour of the cells. Collagen fibers are semi-flexible and form anisotropic networks. The filament contour length L_c is of approximately the same order of magnitude as the persistence length L_p. The persistence length of collagen fibers varies from $L_p \sim 14 - 180$ nm, while the contour length is $L_c \sim 309$nm[39].

The viscoelastic properties of collagen-I networks can be examined over four distinct time regimes that reflect expected structural changes within the network: (i)nanoseconds to milliseconds, associated with intra-filament interactions arising from the conformations of individual fibers; (ii)seconds, related to inter-fiber interactions and the orientation of fibers within mesoscopic domains; (iii)minutes, which involve alterations in the size and shape of these domains; and (iv)tens of minutes to hours, which pertain to the sliding of domains over one another[18,40,41]. The multi-time structural changes of collagen I matrix are shown schematically in **Figure 2**:

Figure 2.

We aim to explore the cumulative effects resulting from structural changes in collagen I networks occurring over minutes and hours, as these temporal scales are significant in relation to cellular dynamics. Tractions of migrating epithelial collectives on a collagen I matrix induce gradual increase in matrix strain. Each increment in strain leads to an increase in matrix stress, followed by a stress

relaxation that contributes to the accumulation of residual stress[18]. Consequently, our focus is directed to mesoscopic domains which exist over some period of time and then disappear (**Figure 1c,d**). In experiments without cells, a progressive increase in uni-axial extensional strain (a step strain increment of 0.02) triggers successive stress relaxation cycles[40]. The stress within the matrix tends to relax towards the residual stress. Notably, the residual stress observed after the initial relaxation cycle was approximately 5Pa, which rose significantly to around 35Pa following the third relaxation cycle in response to repetitive stress applications[40]. The relaxation time corresponds to minutes. It is a consequence of the rearrangement of filaments within mesoscopic collagen domains under applied strain caused by cumulative effects of inter- and intra-filament interactions. A similar result was obtained by considering stress relaxation under shear strain[41]. The corresponding constitutive model for the long-time viscoelasticity of collagen I networks caused by remodelling of domains and domain sliding could be also the Zener model already presented in **Table 1**.

Clark et al.[4] demonstrated that cell clusters apply asymmetric radial traction forces directed inward at the edges of a cluster. This action leads to an in-plane bi-axial extension of the thin collagen network in the areas surrounding the cells, while simultaneously causing in-plane bi-axial compression directly under the cell cluster, as shown in **Figure 3**:

Figure 3.

Cells exert mechanical forces on the extracellular matrix during movement, typically ranging from 10 to 100nN[42]. Notably, mesenchymal cells generate greater traction forces than epithelial cells[34]. These forces significantly exceed the threshold necessary to disrupt electrostatic and hydrophobic interactions within collagen I networks, estimated to be around 20pN[41].

It is interesting to discuss these structural changes of a collagen I network in the context of its stiffness. Cell tractions generate strain, which induces change in the energetic state of collagen mesoscopic domains $\langle e_c \rangle^{CN}$. If we suppose that the Zener model (**Table 1**) can describe the viscoelasticity of collagen networks over minutes and hours, the following conditions should be satisfied: (i) $\langle e_c \rangle^{CN}(r, t + \Delta t, \tau) = \langle e_c \rangle^{CN}(r, t, \tau) - \Delta W_d$ and (ii) $\langle e_c \rangle^{CN}(r, t_{eq}, \tau + \Delta\tau) = \langle e_c \rangle^{CN}(r, t_{eq}, \tau) + \Delta W_s$.

The short-term change in the energy $\langle e_c \rangle^{CN}$ after the increment of time Δt depends primarily on the density of established inter-fiber bonds ρ_B and the effective radii of mesoscopic domains. It can be expressed as:

$$\langle e_c \rangle^{CN}(r, t + \Delta t, \tau) = \langle e_c \rangle^{CN}(r, t, \tau) + \left(\frac{\partial \langle e_c \rangle^{CN}}{\partial R_{eff}}\right)_{\rho_B} \frac{dR_{eff}}{d\tau} + \left(\frac{\partial \langle e_c \rangle^{CN}}{\partial \rho_B}\right)_{R_{eff}} \frac{d\rho_B}{d\tau} \quad (3)$$

where R_{eff} is the effective radius of a mesoscopic domain and ρ_B is the density of inter-fiber bonds.

The long-term change in the energy $\langle e_c \rangle^{CN}$ after the increment of time $\Delta\tau$ depends mainly on the packing density of collagen fibers within the domain and sliding speed of the domains. It can be expressed as:

$$\langle e_c \rangle^{CN}(r, t_{eq}, \tau + \Delta\tau) = \langle e_c \rangle^{CN}(r, t_{eq}, \tau) + \left(\frac{\partial \langle e_c \rangle^{CN}}{\partial n_{CN}}\right)_{T_{eff}^F} \frac{dn_{CN}}{d\tau} + \left(\frac{\partial \langle e_c \rangle^{CN}}{\partial T_{eff}^F}\right)_{n_{CN}} \frac{dT_{eff}^F}{d\tau} \quad (4)$$

where $n_{CN}(r, \tau)$ is the packing density of fibers equal to $n_{CN}(r, \tau) = \sum_i (r - r_i(\tau))$, r_i is the centre of mass of the i-th fiber, and T_{eff}^F is the effective temperature given by: $k_B T_{eff}^F = \frac{D_F}{\mu_F}$[30], D_F is the diffusivity of domains, and μ_F is the mobility of domains.

Bi-axial in-plane extension and compression of the collagen I network, caused by cell tractions, influences energy storage and energy dissipation. Energy storage is directly correlated with the matrix

residual stress and consequently with the matrix stiffness[18]. Extension leads to short-term energy dissipation caused by disruption of inter-chain bonds, while the effective radii of domains increase. Bi-axial compression induces more intensive disruption of inter-filament bonds in comparison with the bi-axial extension due to filaments stretching. The effective domain radius decreases under compression.

As a result, it can be anticipated that more intensive short-term energy dissipation will take place during compression than during extension, leading to the bending and buckling of filaments, along with a perturbation in the alignment of filaments. This significant energy dissipation contributes primarily to the softening of domains when subjected to compression, whereas these domains exhibit considerably greater stiffness when extended, a phenomenon influenced by enthalpic and entropic factors during the stretching of filaments.

Long-term effects of the rearrangement of a collagen network are associated with alterations in the packing density of filaments and their relative movement over one another. Extension stimulates domain sliding, while compression reduces it. Consequently, intensive domain sliding accompanied by stiffening of domains themselves caused by stretching of the fibers lead to matrix stiffening, while disordering of the matrix caused by compression is accompanied by softening of the domains.

In further consideration, we will discuss the impact of matrix stiffening on the stiffness of cell monolayers under in vivo and in vitro conditions, and the impact of matrix viscoelasticity on collective cell migration.

4. Change of cell stiffness caused by changing the stiffness of the substrate matrix

A cell's mechanosensing of the stiffness of the substrate matrix depends on its number of contractile units [1]. Research by Yang et al.[33] and Tijore et al.[34] has indicated that mesenchymal cells exhibit a diminished ability to sense the stiffness of a matrix. Conversely, epithelial MCF10A cells demonstrate a strong capability to form contractile units (CUs), whereas mesenchymal cancer cells, such as MDA-MB-231, are deficient in the cytoskeletal protein tropomyosin 2.1, which is essential for effective rigidity sensing[34]. Observations of human foreskin fibroblasts (HFF) revealed the formation of 39CUs per 100 μm² on rigid pillars (k = 8.4pNnm–1) and 24CUs per 100μm² on soft pillars (k = 1.6pNnm–1) within a 10-minute period during the initial spreading phase. In stark contrast, mesenchymal cells generated fewer than 2CUs per 100μm² in the same timeframe on both rigid and soft pillars[33]. In vitro investigations suggest that epithelial cells exhibit increased stiffness while migrating on stiff substrates, yet display a softer phenotype when cultured on compliant surfaces, which are generally regarded as non-permissive for migration. It is in accordance with fact that the number of contractile units (CUs) increases on stiffer substrates, which enables cells to store more contractile energy. Stiffer substrates also stimulate: reinforcement of cell-cell adhesion contacts, and cell migration[1, 10]. The cell-substrate contact area and cell tractions also increase with substrate stiffness[1].

Marchant et al.[3] demonstrated that neural crest cells organized in clusters from Xenopus embryos exhibit a dynamic reduction in stiffness in response to the temporal stiffening of their native substrate, specifically the mesoderm layer, which is crucial for initiating collective cell migration. The stiffness of these cell clusters was assessed through the apparent elastic modulus, utilizing atomic force microscopy. In the context of mesenchymal cells, including neural crest cells, a stiffer substrate promotes the expression of mechanosensitive Piezo1 channels while simultaneously diminishing the formation of CUs[34]. This phenomenon results in decreased contractility of these cells, rendering them softer. The activation of Piezo1 channels leads to an increase in calcium influx, which in turn

induces microtubule deacetylation. Marchant et al.[3] identified microtubule deacetylation as a key factor contributing to the softening of the neural crest cell population when situated on a stiffer mesoderm substrate.

Accurate regulation of the formation and dissolution of integrin-mediated FAs is crucial for efficient cell migration, with the disassembly process being reliant on calcium influx [43]. Yao et al.[44] emphasized the importance of mechanosensitive Piezo1 channels in the modulation of FAs. The influx of calcium not only impacts the stability of FAs but also affects the integrity of cell-cell adherens junctions(AJs). Key characteristics of mesenchymal cells that contribute to their mechanosensitivity, distinguishing them from epithelial cells, include: (i)relatively weak N-cadherin-mediated cell-cell AJs, (ii)smaller FAs[34,44], (iii)reduced cell contractility[44], (iv)a softer cytoskeleton[34], and (v)externally-induced fluctuations in intracellular calcium levels[45].

5.Matrix viscoelasticity and collective cell migration

The processes through which cells detect variations in viscoelasticity are just beginning to be understood; however, their reactions to mechanical environments are extensively documented and often exhibit significant complexity. Stiffness associated with energy storage represents only one aspect of the viscoelastic properties of substrate matrices. Another crucial aspect pertains to energy dissipation and the rate at which it occurs. Substrate matrices that meet the following criteria: (i)stress can relax towards the equilibrium residual stress, (ii)viscoelastic behaviour can be modelled using a linear constitutive framework, and (iii)residual stress is entirely elastic, can be characterized by the specific properties:

- Constant values of the storage energy and corresponding matrix stiffness are obtained under constant strain conditions,
- Energy dissipation changes during stress relaxation from an initial maximum value to the zero obtained at equilibrium leads to matrix softening, and
- The rate of energy dissipation depends on the stress relaxation time.

A reduction in the stress relaxation time enhances the rate of energy dissipation. Adebowale et al.[2] demonstrated that single-cell migration on two-dimensional stiff elastic substrates is primarily mediated by lamellipodia at the leading edge. In contrast, migration on viscoelastic substrates occurs independently of lamellipodia and is instead facilitated by filopodia. Lamellipodia are characterized by branched networks of actin, while filopodia consist of elongated, unbranched actin filaments. The softer and more flexible nature of filopodia makes them better suited for migration on substrates exhibiting time-dependent stiffness which can be correlated with the stress relaxation time within a substrate matrix caused by cell migration.

Both rapid, and very slow, stress relaxation within a matrix promotes cell migration[2,46]. Rapid stress relaxation, characterized by a relaxation time of several seconds, facilitates an extended period during which the equilibrium value of the storage elastic energy and matrix residual stress remains stable under constant strain conditions, thereby supporting cellular adaptation and the maturation of FAs[2]. Conversely, slow stress relaxation, with a relaxation time spanning from several tens of minutes up to hours, also creates favourable conditions for cellular adaptation[46,47]. Charbonier et al.[46] consider collective migration of MDCK monolayers on the substrate matrices with a Young's modulus of 20kPa and various stress relaxation times between of 45s and 11.7min. The slow stress relaxation time ensures: (i)formation of leader cells, (ii)lamelopodia-mediated cell migration, and (iii)establishment of supracellular actin cables. All contribute to the efficiency of cell migration[46]. However, a matrix

stress relaxation time of minutes reduces cell migration. This observation aligns with the understanding that the growth rate of FAs is highest within the first 2min[48]. Huerta-López et al.[47] pointed out that a substrate matrix with a Young's modulus of 20kPa and two relaxation times, i.e. one of $\sim 10 - 20$s and the other of a few hours ensured the largest FAs of RPE-1 cells.

6. Conclusion

Mechanobiology has consistently concentrated on the various mechanisms by which cells respond to stiffness, and stiffness gradients, in cellular systems and substrate matrices. The term "stiffness," commonly used to characterize the material properties of both cellular structures and substrates, lacks a definitive interpretation of the term especially in the case of viscoelastic systems. It is well established that multicellular systems and substrate matrices exhibit viscoelastic behaviour, indicating that stiffness and stiffness gradients are dependent on both spatial and temporal factors, with changes occurring over a range of time scales. Our discussion centres on two types of monolayer: epithelial and mesenchymal monolayers, as well as a collagen I matrix. We present our findings, which are based on a combination of experimental and theoretical analyses, as follows:

- Viscoelasticity is characterized by the structural alterations of a system in response to applied strain or stress, whether from external or internal sources. Changes in the loading conditions may require modifications to the constitutive model that describes viscoelastic behaviour. The resulting structural changes lead to energy storage and energy dissipation, which can occur either simultaneously or on varying time scales, depending on the specific constitutive model utilized.
- The constitutive model that describes viscoelastic behavior resulting from collective cell migration is influenced by the density of cell packing as well as the intensity of adhesion interactions between cells, and between cells and the substrate matrix. Variations in cell packing density arise due to the mechanical stresses that are typically produced during cell migration.
- The stiffness of a system is influenced by the interplay between elastic energy storage, which contributes to system stiffening, and energy dissipation, which leads to system softening. As a result, the stiffness of the system varies over time. The rate at which the stiffness of multicellular systems changes elicits diverse cellular responses, occurring over the span of minutes up to hours.
- The storage of elastic energy in cellular systems necessitates robust inter-cellular connections facilitated by strong cell-cell adhesion contacts. A reduction in the strength of these adhesion contacts is primarily responsible for energy dissipation and the softening of the system, even in instances where cells increase their contractility and, as a result, become stiffer.
- Cellular traction forces lead to alterations in the structural configuration of the collagen I matrix, thereby affecting its stiffness and subsequently influencing cell migration. These structural modifications within the collagen matrix occur over a range of temporal scales. Changes arising within minutes are associated with the alignment of filaments and the cumulative impact of their conformational adjustments under strain, which is accompanied by bending and buckling phenomena within mesoscopic domains. In contrast, the sliding of these domains relative to one another takes place over the course of several hours. Domain stiffening, along with the establishment of connectivity between filaments, is essential for the overall stiffening of the matrix.

- The stiffness of the extracellular matrix affects the stiffness of monolayers in various ways, largely contingent upon the specific cell type involved. Epithelial cells exhibit a greater capacity to adjust to alterations in matrix stiffness, as compared to cancerous mesenchymal-like cells.
- The rate of energy dissipation within the collagen matrix is influenced by the stress relaxation time of the matrix. Either a more rapid energy dissipation, or a very slow energy dissipation towards equilibrium, facilitate an extended quasi-static time-period of matrix suitable for the maturation of FAs, which is essential for effective cell migration.

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Figure captions:

Figure 1. Schematic representation of the physical parameters that influence energy storage and dissipation, and their inter-relationships

Figure 2. The multi-time structural changes of collagen I matrix caused by externally applied stress or strain influences the matrix viscoelasticity.

Figure 3. Cell tractions during collective cell migration induce in-plane and out-of-plane strain within a collagen I matrix. Radial cell tractions, indicated by black arrows, influence the orientation of collagen fibers and alter their density, resulting in a higher concentration of fibers in the area directly beneath the cell cluster.

Table captions:

Table 1. Viscous and elastic parts of stress for various viscoelastic models accompanied by energy storage and dissipation.

Table 2. Changes in model parameters under extension/compression cause energy storage and dissipation, leading to change in the stiffness of migrating epithelial collectives.