

**Engineering dynamic extracellular
microenvironments:
Temperature-casted Collagen-Fibronectin scaffolds
with controlled structure, mechanics, and
cell-driven re-organisation**

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Abstract

Understanding how to engineer biomimetic scaffolds is critical for advancing tissue engineering, regenerative medicine, and disease modelling. This thesis investigates the design, fabrication, and characterization of three-dimensional (3D) extracellular matrix (ECM) scaffolds, focusing on collagen–fibronectin (ColFn) hydrogels that closely replicate native ECM environments.

The introductory chapters establish the fundamental principles of tissue engineering, emphasizing the role of collagen and fibronectin in ECM mechanics, cellular interactions, and mechanotransduction. Building upon this framework, the thesis integrates advanced confocal microscopy and oscillatory rheology to enable high-resolution, real-time imaging and precise mechanical analysis of ECM scaffolds.

A temperature-controlled fabrication technique was developed to modulate scaffold architecture and mechanical properties. Incorporating fibronectin significantly enhanced scaffold stability, fibre organization, and cellular engagement, supporting dynamic reorganization processes essential for tissue regeneration. The experimental work highlights how fibronectin modulates collagen fibre formation kinetics, increases ECM stiffness, and promotes cell adhesion and migration.

Live imaging and rheological analyses revealed that fibroblasts dynamically remodel their microenvironment, forming pericellular collagen–fibronectin micro-islands and aligning fibres along force axes. Importantly, a critical cell concentration was identified, beyond which collective cell-generated forces induced a phase transition in the ECM. At low cell densities, cells contributed to local stiffening and retained more space to spread and interact with their surroundings. However, as cell density increased, mechanical stress and crowding altered ECM behaviour, transforming the structure from a continuous fibrous network into distinct, clustered islands of collagen and fibronectin. This structural shift was accompanied by a substantial increase in ECM stiffness, reflecting how cell density and mechanical feedback collectively govern matrix architecture and mechanical properties. While these observations parallel aspects of natural tissue remodelling, they are unique insights into the phase transition behaviour of engineered ECM under cellular stress.

Overall, this thesis provides a comprehensive framework for understanding scaffold design, ECM reorganization, and cellular interactions under physiologically relevant conditions. By integrating scaffold fabrication methods with mechanobiological insights, the findings contribute to the development of next-generation, bioactive materials for regenerative medicine and tissue engineering applications.

Statement of Originality

Unless stated otherwise, the work presented in this thesis was performed by the author during the duration of their degree under the supervision of Dr. Delphine Gourdon and Dr. James L. Harden. All experimental work was performed by myself. All data analysis, including the coding used for image analysis, was also performed by myself. Data interpretation and final conclusions were performed by myself in consultation with Dr. Delphine Gourdon and Dr. James L. Harden. Chapters 4, 5, and 6 of this thesis are the basis for several manuscripts in preparation for publication. Results from this work were also presented in a poster at the 7th Annual Meeting of the Biophysical Society of Canada in Ottawa in May 2022.

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
α -MEM	Minimum Essential Medium Eagle - alpha modification
AFM	Atomic Force Microscopy
ANOVA	Analysis of variance
Col	Collagen
DMA	Dynamic Mechanical Analyzer
ECM	Extracellular matrix
FBS	Fetal bovine serum
Fn	Fibronectin
LVR	Linear Viscoelastic Region/Regime
PBS	Phosphate-buffered saline
VEGF	Vascular endothelial growth factor

Chapter 1

Foundations of Tissue Engineering and ECM Dynamics

1.1 Introduction to tissue engineering

In 2022, over 157,000 organ transplants were performed worldwide, yet more than 103,000 patients remained on waiting lists, highlighting the persistent gap between organ demand and availability [1]. In Canada (according to the 2023 CIHI transplant report), 6997 individuals required a transplant in 2023, and 216 people died while waiting for one [2] (Table 1.1). Traditional solutions such as autografts (using tissue from another part of the same body) and xenografts (from other species) have shown some success, but their application is limited by immune rejection, donor site morbidity, and other complications [3, 4]. Despite advances in surgical and immunological techniques (Figure 1.1), these limitations continue to drive research toward alternative strategies, particularly tissue engineering approaches.

Table 1.1: Organ Transplant Statistics for Canada in 2023

Organ	Transplants	Waiting List	Deaths
Kidney	2,078	2,522	91
Liver	666	509	79
Heart	184	132	13
Lung	450	179	30
Pancreas	112	165	3

Tissue engineering has gained increasing attention for its potential to alleviate organ shortages by enabling the development of lab-grown tissues and organs. This emerging field integrates methods from biology, physics, and medicine to create biological substitutes that can replace, repair, or enhance damaged tissues [5]. Some of its earliest clinical successes include engineered human skin grafts, which are now routinely used in burn and wound treatment [6, 7, 8]. Achieving similar success with more complex tissues requires a deep understanding of both cellular behaviour, such as proliferation, migration, and differentiation, and the structural and biochemical properties of scaffolds (engineered 3D biomaterial structures that support cell adhesion, growth, and tissue formation), including their material composition and microarchitecture [4].

Tissue engineering typically follows a multi-step design process. It begins with the isolation of cells from a donor tissue source (Figure 1.2), followed by in vitro expansion. These cells are then seeded onto a scaffold that provides a structural framework and biochemical cues necessary for tissue development. After an initial period of maturation, the cell-scaffold construct may be implanted into the body to promote regeneration.

Successful tissue engineering depends heavily on understanding how cells interact with their scaffold environment. The scaffold's material properties, architecture, and biochemical signals all

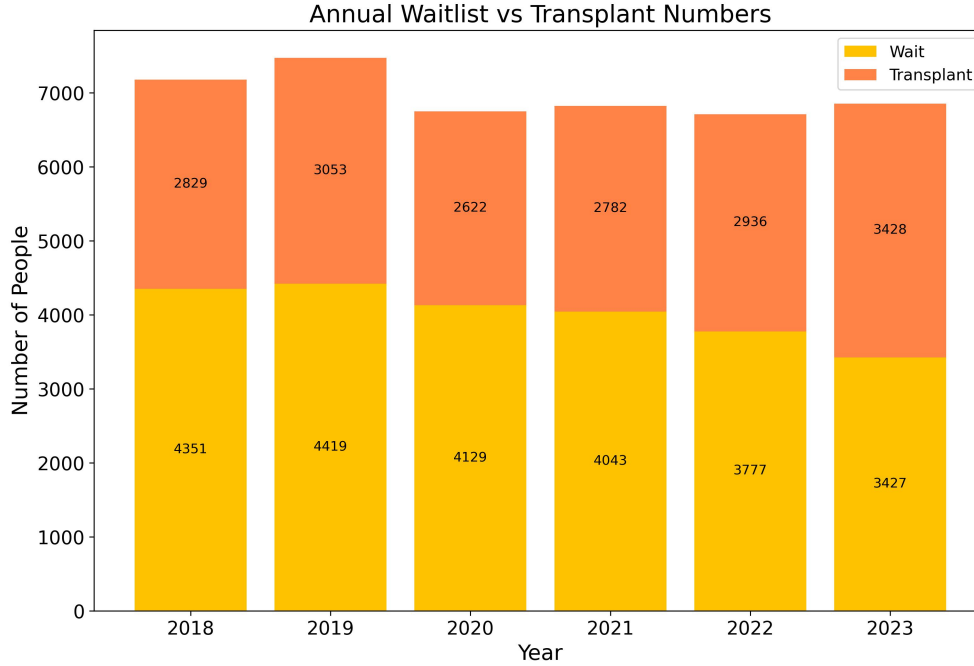


Figure 1.1: Stacked Bar Chart of Annual Waitlist versus Transplant Numbers in Canada from 2018 to 2023. This chart illustrates the comparison between the number of patients on the waitlist (yellow bars) and those who received transplants (orange bars) each year. Despite fluctuations, a growing trend in transplants can be observed in 2023, indicating some progress in addressing the backlog of patients awaiting organ transplants.

influence cellular behaviour, including adhesion, proliferation, and differentiation. The following section explores these scaffold-related parameters in more detail, with a particular focus on the composition, structural characteristics, and dynamic interactions between scaffold components and different cell types.

As tissue engineering continues to evolve, increasing attention is being paid to scaffold materials that not only mimic the structural complexity of the native extracellular matrix (ECM) but also support dynamic interactions with cells. Natural ECM proteins such as collagen and fibronectin have shown particular promise in this regard, offering both mechanical support and integrin-binding sites essential for cellular communication. A deeper understanding of how these components influence cell behaviour is critical for designing effective, biologically active scaffolds. The next section explores the structure and function of the ECM, emphasizing the roles of collagen and fibronectin in supporting tissue regeneration.

1.2 Extracellular Matrix

In all tissues and organs, the ECM serves as the non-cellular component, providing structural and biochemical support to surrounding cells [9]. The ECM is a three-dimensional (3D) network consisting of glycoproteins, polysaccharides, and proteoglycans (PGs) [10]. However, the majority of ECM is made up of water, proteins and its structure and composition can vary greatly between different tissues. For instance, the ECM of cartilage includes a dense network of PGs and collagens, providing cartilage with its compression-resistant properties [11]. In contrast, the ECM in the kidneys forms a basement membrane, a thin sheet of fibers enriched with glycoproteins such as laminin, entactin,

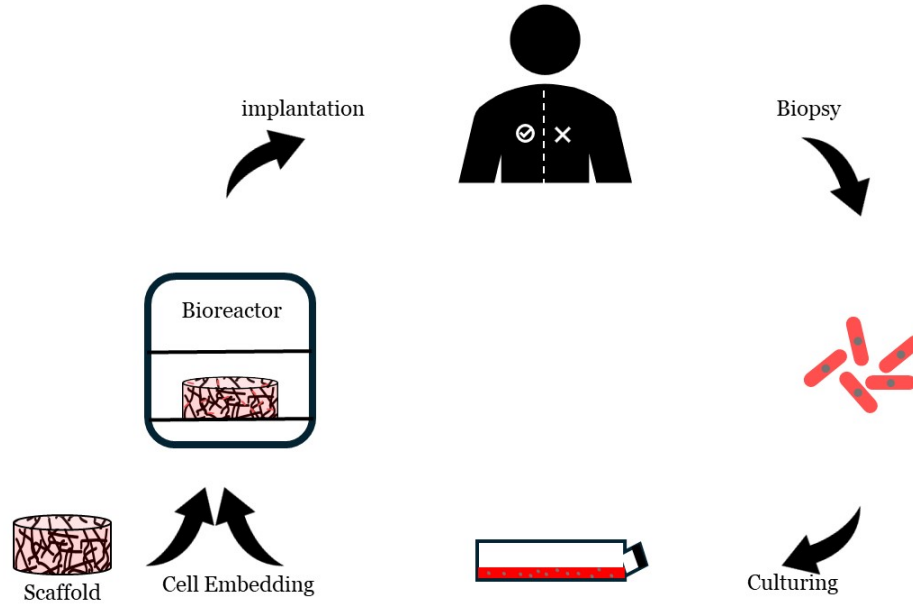


Figure 1.2: Tissue Engineering Process. *This schematic illustrates the key stages in tissue engineering: starting with a biopsy to obtain cells, followed by cell culturing, embedding the cells into a scaffold, and using a bioreactor to prepare the scaffold for implantation into the human body.*

and collagen, which facilitates molecular filtration [12].

There is a continuous and mutual interaction between the ECM and cells, leading to ongoing changes in the ECM's composition such as shifts in mechanical properties, pH, oxygen concentration, and other variables in the microenvironment based on cellular activities and environmental conditions [13]. These changes, in turn, influence cell behaviour, creating a constantly adapting structure-function relationship that makes the ECM an ideal scaffold for supporting cell populations [8, 14, 15]. This dynamic interaction is essential for maintaining tissue homeostasis and responding to physiological needs, making it a critical concept in understanding tissue biology and engineering.

Furthermore, abnormalities in the ECM are directly linked to diseases such as cancer. Genetic abnormalities in ECM proteins can lead to a wide range of syndromes, which can be anything, from minor to severe [16]. To create functional tissues and organs, it is essential to replicate the diverse ECM compositions and organizations found in native tissues and understand their behaviour in the presence of cells. Achieving this goal requires studying factors that impact the ECM, including key proteins and materials within tissues, and understanding how cells interact with these components. This comprehensive understanding enables the replication of the complex interactions between cells and their ECM, which is crucial in tissue engineering, where one may desire to mimic the biological recognition molecules that control the relationships between cells and their ECM.

The ECM has two main roles in tissues: to provide structural support and signalling functions to cells [17, 14, 15]. The structural function of scaffolds is to organize cells in a specific 3D shape and give the construct mechanical properties that match the in vivo tissue architecture. The signalling properties of scaffolds are to allow cells to attach, survive, and in some cases perform advanced, tissue-specific functions. Several studies have been shown that the physical properties of ECM affect cell interactions such as cell migration [18], spreading [19], differentiation [20] and proliferation [21]. In addition to this, the ECM serves at least three functions in its role controlling cell behaviour: it provides adhesion signals, it provides growth factor binding sites, and it provides degradation sites to give way to the enzymatic activity of cells as they migrate [22]. These properties are discussed separately in the following sections, including an examination of the ECM's mechanical functions,

the ECM proteins, and the ECM’s role in cell signalling.

The ECM is far more than a structural filler; it plays an active and dynamic role in regulating cellular behaviour through both mechanical and biochemical signalling. Its architecture and composition are central to tissue development, repair, and function. As such, understanding the ECM is fundamental to tissue engineering, where the goal is to recreate or influence these complex biological environments. In the context of scaffold design, particular attention must be paid to ECM components that not only provide mechanical strength but also interact directly with cells. Collagen and fibronectin, two such critical proteins, form the foundation of the engineered scaffolds studied in this thesis and are explored in further detail in the following sections.

1.3 Dynamic Mechanical Functions of the ECM

To effectively design functional tissue scaffolds, it is essential to understand the native ECM, a complex and dynamic structure that regulates not only mechanical stability but also cell signalling and fate decisions. The following sections will explore these structural and biological roles in greater detail.

The ECM plays a critical role in determining the mechanical properties of tissues, influencing their rigidity, elasticity, and ability to withstand various mechanical forces [23]. In bone, the ECM is predominantly composed of collagen I, deposited in a lamellar pattern. As bone matures, a significant portion of the ECM becomes calcified, which imparts mechanical rigidity and load-bearing strength essential for bone’s structure [24, 25]. Meanwhile, the uncalcified organic portion of the ECM provides elasticity and flexibility, allowing bones to absorb and dissipate dynamic loads effectively [26]. In cartilage, the ECM’s primary components are PGs, such as aggrecans, which are heavily glycosylated and hydrophilic [27]. These PGs attract water molecules, creating a gel-like structure that enables cartilage to endure compressive loads while maintaining durability through repeated reversible deformation. This viscoelastic property is crucial for cartilage to function as a shock absorber in joints, protecting bones from mechanical stress during movement [28].

Ligaments and tendons also rely on their ECM for mechanical function but in different ways (Figure 1.3). Ligaments, composed mainly of collagen I, are structured to withstand tensional forces [29, 30]. The ordered arrangement of collagen fibers in ligaments aligns with the direction of tensile stress, providing strength and stability to joints [31, 32]. In contrast, tendons, which connect muscles to bones, consist of parallel collagen fibers with orthogonal proteoglycan bridges [32]. This organization allows tendons to efficiently transmit tensile stresses from muscles to bones, ensuring effective force transfer during muscle contraction [30, 32, 33].

The ECM’s dynamic nature further enhances its mechanical functions. It is not merely a static scaffold but undergoes constant remodelling through cell-mediated processes. This reorganization allows the ECM to adapt to changing physiological conditions and maintain tissue homeostasis [35]. Changes in the ECM’s microarchitecture, particularly in collagen networks, can mechanically regulate cell behaviour and interactions [36]. For instance, increased ECM protein deposition can lead to stiffening of the ECM, affecting tissue mechanics and cell function [37].

In pathological conditions, such as tumors, ECM remodelling and abnormal collagen deposition can result in increased tissue stiffness. This altered mechanical environment can impact cell behaviour, including differentiation, migration, and proliferation, highlighting the importance of ECM integrity in maintaining normal tissue function [38, 39]. The ECM’s composition, organization, and dynamic remodelling are fundamental to its role in providing mechanical support to tissues, ensuring that tissues can withstand various mechanical forces, maintain structural integrity, and support cellular functions critical for overall tissue health [39].

The mechanical behaviour of the ECM, particularly its ability to undergo viscoelastic deformation and respond to cellular forces, is central to tissue function and regenerative processes. These dynamic properties not only influence how tissues withstand mechanical loads but also regulate key cellular behaviours through mechanotransduction. For tissue engineering applications, especially in the development of responsive biomaterials, it is essential to replicate this functional interplay between cells and their matrix. The design of collagen-based scaffolds, as explored in this thesis, seeks to

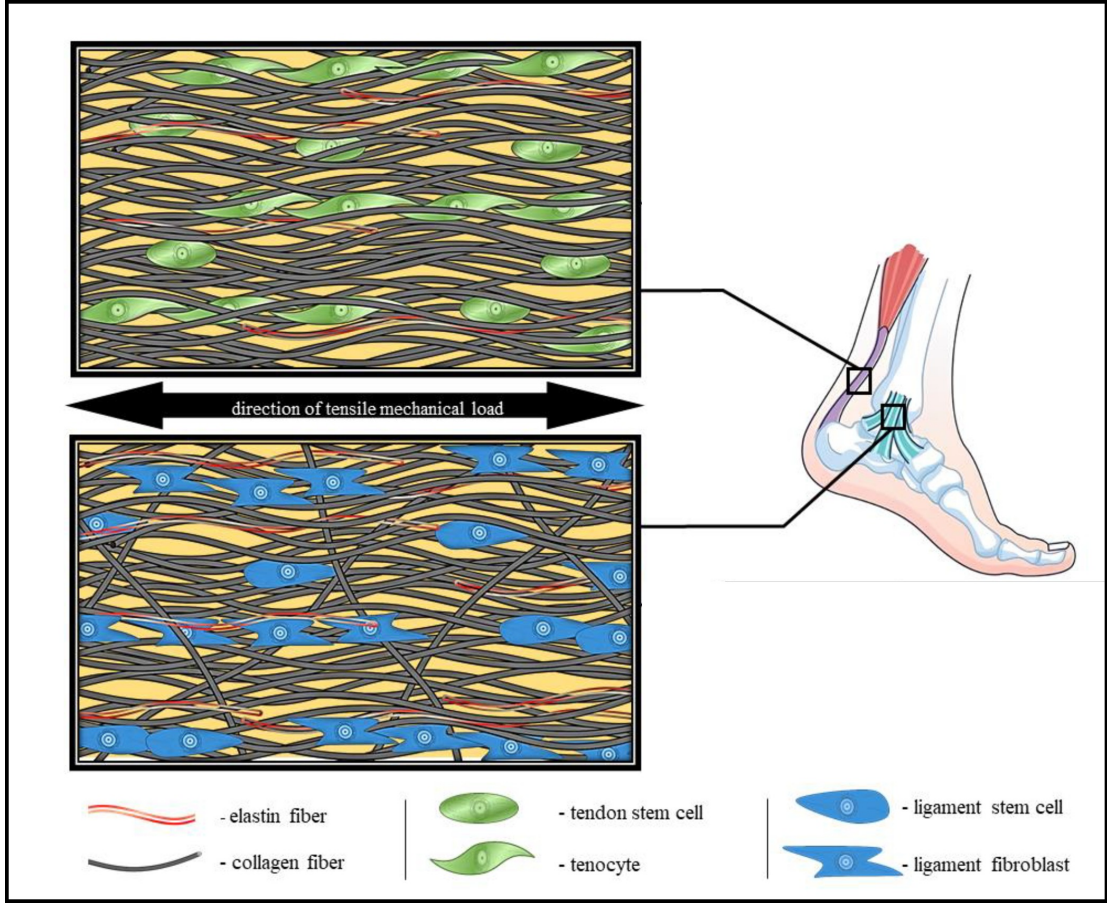


Figure 1.3: Comparison of molecular structure, fibers organization and composition of tendon and ligament [34]. *Ligaments exhibit a moderately interwoven collagen network that accommodates multi-axial joint motion, whereas tendons display highly parallel collagen bundles aligned with uniaxial tensile loads. These contrasting ECM architectures underlie the different mechanical roles of ligaments and tendons in transmitting forces and stabilizing joints.*

capture these dynamic mechanical characteristics by tuning scaffold composition and fabrication conditions.

1.4 ECM interaction with cells

The ECM serves as a crucial mediator of cell signalling, influencing a range of cellular processes from proliferation to differentiation and migration. Understanding how ECM components affect these processes provides insight into its role in tissue development, maintenance, and repair. For instance, it impacts cell proliferation, as evidenced by studies showing that ECM components like fibronectin and collagen influence cell growth and cancer risk [38]. In vitro, experiments have demonstrated that ECM proteins such as fibronectin regulate cell proliferation, differentiation, migration, survival, and cytoskeletal organization [20, 21, 40, 41, 42]. The ECM's interaction with cellular signalling pathways is integral to its function [43, 44]. ECM components, such as fibronectin, have been shown to regulate vascular remodelling and connect muscle contraction with local vasodilation in vivo experiments [43, 44]. These interactions are not limited to vascular tissues; they are also critical during embryonic development, where the ECM facilitates mesenchymal condensation and self-assembly processes essential for skeletogenesis [45, 46]. This broad spectrum of influence underscores the ECM's capacity to dictate cellular behaviour by providing structural and biochemical cues.

These interactions between cells and the ECM are governed by a variety of membrane-bound receptors that mediate both mechanical and biochemical communication. Through these receptors, cells sense ECM stiffness, bind to specific matrix components, and initiate downstream signalling cascades that regulate behaviours such as adhesion, migration, and remodelling. The next section reviews the major classes of cell surface receptors involved in these processes, with a focus on those most relevant to ECM engagement and scaffold-based applications.

These interactions between cells and the ECM are not passive; they involve continuous feedback in which cells sense, reorganize, and adapt their surrounding environment. Such dynamic reciprocity plays a critical role in tissue development and repair, and is particularly important in engineered systems that aim to replicate physiological behaviour. Understanding how specific ECM components influence this interaction is essential for guiding scaffold design, both in terms of mechanical performance and biological functionality. With this in mind, the following sections examine the molecular and structural roles of two key ECM proteins, as well as the integrin-mediated receptor mechanisms that enable cells to engage with and remodel their extracellular environment.

1.5 Integrin

Interactions between cells and the ECM are mediated by cell-surface glycoprotein receptors, particularly those that bind to ECM proteins and regulate adhesion, migration, and signalling pathways [47, 22]. These receptors belong to a group collectively referred to as Cell Adhesion Molecules (CAMs), which include cadherins, selectins, immunoglobulins, and integrins [22]. While the first three primarily mediate cell-cell adhesion, integrins are crucial for both cell-cell and cell-ECM interactions and are the most relevant to this study.

Cadherins, selectins, and immunoglobulin-like CAMs function largely in homophilic or heterophilic cell-cell recognition processes and are involved in processes such as tissue integrity, inflammation, and immune cell trafficking [48, 49, 50]. However, these receptors do not directly participate in interactions with ECM proteins like collagen or fibronectin and are thus beyond the scope of this work.

Integrins are crucial transmembrane receptors that facilitate ECM adhesion and are involved in bidirectional signal transmission between the ECM and the cell's interior [51]. This family play an important role in embryogenesis, wound healing, and immune response and the behaviour of malignant cells [47, 52].

Structurally, integrins are heterodimeric proteins composed of α and β subunits that non-covalently associate to form functional receptors [10]. Each integrin subunit consists of a cytoplasmic domain, a single membrane-spanning helix, and an ectodomain with a ligand-binding surface [53]. The extracellular domains of these subunits bind to ECM proteins in a cation-dependent manner, involving ions like Ca^{2+} , Mg^{2+} , and Mn^{2+} [10]. These cations are essential for the binding process, specifically at the metal-ion-dependent adhesion site (MIDAS), which stabilizes the interaction between integrins and their ligands [54].

Integrins are not only vital for structural adhesion but also play a significant role in signalling pathways that regulate various cellular behaviours, including migration, proliferation, and survival [47]. Upon binding to their ligands, integrins cluster together to form focal adhesions [55, 56, 57, 58]. These focal adhesions act as signalling hubs, connecting the ECM to the cytoskeleton via adaptor proteins such as talin, vinculin, and α -actinin [22, 59]. This connection facilitates the mechanical stability of cells and the transmission of extracellular signals to the intracellular environment, thereby influencing cellular responses and behaviour [59]. Different subfamilies of integrins, each associated with distinct β subunits, determine their specific functions and ligand specificities [22, 52, 54]. For example, β_1 integrins are primarily involved in cell-matrix interactions with ECM components like collagen, laminin, and fibronectin [47]. In contrast, β_2 integrins, also known as leukocyte integrins, are essential for immune responses, mediating cell-cell interactions among leukocytes [47]. The β_3 integrins, which include the platelet glycoprotein IIb/IIIa, are crucial for platelet aggregation and blood clotting [47].

The interaction between integrins and ECM proteins triggers various intracellular signalling

pathways. These pathways involve the activation of small guanosine triphosphatases (GTPases) such as the Ras homologous (Rho) family, which regulate the formation of actin stress fibers and the assembly of focal adhesions [60, 61]. This process is critical for the mechanical transduction of signals and the organization of the cytoskeleton [62, 61]. Activation of Rho GTPases leads to the formation of actin stress fibers, which are bundles of actin filaments that help maintain cell shape and enable cell movement. The assembly of focal adhesions, complex multi-protein structures, anchors the cell to the ECM and acts as a scaffold for the assembly of intracellular signalling complexes and the attachment of the integrin complex to the F-actin cytoskeleton [22, 60]. Morphological hallmarks of integrin binding to adhesion proteins in the ECM include cell spreading, extension of cellular membrane processes to within approximately 20 nm of the ECM surface, clustering of integrin receptors at the sites of focal contacts, and the assembly of intracellular accessory proteins such as talin, vinculin, and α -actinin at the site of clustered integrins [22]. These sites of clustered receptors and interactions between transmembrane receptors and the intracellular cytoskeleton carry most of the stress between the cells and the ECM or artificial surfaces. Both theoretical analysis and experimental results demonstrate that without the formation of focal contacts and the connection of numerous transmembrane integrin $\alpha\beta$ heterodimer complexes into much larger multi-integrin complexes, cell adhesion would be significantly weaker. This interaction is essential for translating extracellular signals into cellular responses [22].

Additionally, integrin-mediated signalling can influence the organization and assembly of the ECM itself, demonstrating the dynamic and reciprocal nature of integrin-ECM interactions [60, 63]. When integrins bind to ECM proteins, they not only transmit signals into the cell but also affect the composition and structure of the ECM [63]. For example, integrin binding to fibronectin can enhance fibronectin matrix assembly, thereby strengthening the ECM and modifying its mechanical properties [64]. This bidirectional communication ensures that cells and their surrounding matrix are constantly adapting to physiological and environmental changes [64].

The ECM proteins are intricately complex, incorporating multiple functional sites essential for various biological interactions [22]. These proteins have specialized regions for binding to collagen, glycosaminoglycans, and integrins, as well as sites for crosslinking via transglutaminase activity and for protease-mediated degradation [22]. Despite their multifunctionality, the receptor-binding domains specific to integrins are often small oligopeptide sequences, typically less than ten amino acids in length and encompass small fraction of the protein mass [22]. A prominent example is the Arginylglycylaspartic acid (RGD) tripeptide, which, when synthetically replicated and appropriately coupled to a surface, can mimic the adhesive properties of fibronectin. This includes facilitating integrin binding, clustering, focal contact formation, and cytoskeletal organization [65]. The specificity of these receptor-binding sequences is crucial, as even minor alterations in amino acid order, such as changing RGD to Arginylaspartylglycine(RDG), can completely abolish their binding activity [66]. This highlights the precise nature of ECM protein interactions and their critical role in cellular adhesion and signalling. The ECM is often thought of primarily as providing mechanical support for cells, but it actually plays a much more intricate role in cellular behaviour [22]. Beyond mechanical support, the ECM engages in biochemical and biomechanical signalling that profoundly influences cell functions [22]. These interactions, which have been widely studied [67, 68, 69], involve complex biochemical mechanisms that are not yet fully understood [68]. A crucial aspect of ECM signalling is the formation of focal contacts where integrins cluster, leading to the phosphorylation of several proteins, including a key tyrosine kinase known as pp125 focal adhesion kinase (pp125FAK) [22]. This phosphorylation, essential for various cell behaviours such as survival, does not occur if cell spreading is mediated by non-integrin mechanisms. Therefore, the ECM's role extends far beyond structural support, actively participating in regulating cell survival, proliferation, and differentiation through its signalling capabilities [22].

1.6 ECM proteins

As previously discussed, the ECM is a highly complex and dynamic network that plays a critical role in maintaining the structural integrity and functionality of tissues. Composed primarily of fibrous

proteins and PGs, the ECM forms a scaffold that not only supports cells but also regulates their behaviour through biochemical and mechanical cues [70, 71]. This intricate system is organized into two main structural components: the interstitial matrix, which surrounds cells in most tissues, and the basement membrane, a more compact and less porous structure that separates epithelial and endothelial cells from underlying connective tissue [72, 73]. While these two components share some common ECM proteins, they are distinguished by unique combinations and arrangements of ECM polymers, each contributing distinctively to the overall architecture and function of the ECM [74, 75].

At the molecular level, the ECM is primarily made up of fibrous proteins such as collagen, elastin, fibronectin, vitronectin, and laminin [71, 76, 77]. These proteins are responsible for providing tensile strength, elasticity, and structural support to tissues [76].

Collagen, the most abundant protein in the ECM, forms a fibrillar network that gives tissues their strength and rigidity [78, 79]. Elastin, although not discussed in detail here, contributes essential elasticity and resilience to extensible tissues such as skin, lungs, and arteries [79, 80]. Fibronectin and laminin play key roles in cell adhesion and migration, interacting with cell surface receptors to influence cell behaviour and tissue organization [22, 79]. While these proteins contribute broadly to ECM function, this thesis focuses specifically on collagen and fibronectin, two ECM components that play central roles in scaffold mechanics, cell adhesion, and matrix remodelling.

In addition to fibrous proteins, the ECM is rich in PGs, which consist of a core protein covalently linked to glycosaminoglycan (GAG) chains [71]. These molecules fill the interstitial space within the ECM, forming a hydrated gel that resists compressive forces and facilitates the diffusion of nutrients and signalling molecules [70, 79]. PGs such as aggrecan in cartilage or perlecan in the basement membrane are crucial for maintaining tissue hydration, buffering mechanical stress, and regulating the availability of growth factors. The interplay between fibrous proteins and PGs within the ECM creates a highly adaptable and responsive environment that supports a wide range of tissue-specific functions [79, 81, 82].

The ECM's structure and composition are not static; they are continuously remodeled in response to both physiological and pathological stimuli [79]. This dynamic nature enables the ECM to adapt during tissue development, repair, and disease progression [81]. For instance, in bone tissue, mineralization incorporates calcium phosphate crystals into the collagen matrix, enhancing mechanical strength [26, 83]. Conversely, tendon ECM is arranged to efficiently transmit tensile forces through aligned collagen fibers and proteoglycan bridges, which resist overstretching [33]. These examples underscore the ECM's remarkable versatility and emphasize the critical importance of its structure-function relationship. Fibrous proteins, in particular, provide much of the ECM's mechanical framework, influencing everything from elasticity to cell signalling. Their composition and organization are thus central to the ECM's ability to support diverse tissue-specific functions.

In the following sections, detailed emphasis will be placed on collagen and fibronectin, the two key ECM components studied in this work. These proteins are not only central to the architecture and mechanics of the ECM but also modulate critical cell-matrix interactions relevant to scaffold design and mechanobiology.

1.6.1 Collagen

Collagen is the most abundant protein in the ECM and is crucial for maintaining the structural integrity of various tissues in the body [84]. It exists in multiple forms, many of which are multimeric and fibrillar, making collagen a primary component that interacts with and organizes other proteins [84]. These interactions are essential for the adhesion and structural support of cells, facilitated by collagen's binding to integrins, particularly $\alpha1\beta1$ and $\alpha2\beta1$ [84, 85, 86]. This interaction plays a significant role in maintaining tissue architecture and facilitating cellular communication within the ECM [84].

Collagen family are the dominant protein in the ECM, with 28 members, providing distinct mechanical, functional, and physical properties across different tissues [87]. For example, type I collagen is predominant in bone, skin, and tendon, contributing to the tensile strength of these tissues, while type II is critical in cartilage, providing elasticity and resistance to compressive forces [88]. Fibril-forming collagens, particularly types I, II, and III, are the most prevalent among the various

types of collagen [87, 89]. Collagen’s presence is not limited to soft tissues like blood vessels and nerves but extends to more rigid structures like cartilage, ligaments, and bone [88]. This diverse functionality underscores collagen’s importance in both the mechanical properties and biological functions of tissues.

Collagen molecules are typically about 300 nm in length, 1.5 nm in diameter and characterized by their triple helical structure, which is fundamental to their function [90]. This structure is composed of three polypeptide α chains, with the classic example being collagen type I, which contains two $\alpha 1$ chains and one $\alpha 2$ chain [91]. The repeating Gly-X-Y sequence, where glycine is essential for the helix formation, allows the three chains to tightly wind around each other, forming a stable, rigid triple helix [84, 91]. This helix is further assembled into microfibrils, which are then cross-linked to form larger, more robust collagen fibers. These fibers are integral to the structural integrity of various tissues, with their mechanical properties being influenced by their arrangement and degree of cross-linking [84]. For instance, the high stiffness of bone, the compliance of tendons, and the gradient of rigidity in cartilage are all attributed to the specific structural configurations of collagen fibers [84, 90].

Collagen plays a critical role in the biomechanical properties of tissues, with its fibers providing the necessary support for maintaining tissue shape and function [84]. The diameter and cross-linking of collagen fibrils can vary, leading to differences in tissue strength and elasticity [84]. This variability allows collagen to meet the specific mechanical demands of different tissues, whether it’s providing stiffness to bones or elasticity to skin and tendons [84]. Genetic mutations in collagen genes can disrupt these properties, leading to a range of connective tissue disorders [92, 93]. For example, mutations in collagen type I can result in osteogenesis imperfecta, characterized by brittle bones, while mutations in collagen type II are associated with cartilage disorders such as chondrodysplasias. These mutations can affect the assembly, structure, and function of collagen molecules, leading to impaired tissue development and function [94, 95].

Over the past years, our understanding of collagen has been significantly expanded, particularly with the identification of novel fibrillar collagens such as types XXIV and XXVII. These collagens share structural similarities with classical fibrillar collagens but also exhibit unique features, including shorter triple helices and specialized tissue localization [84]. For example, collagen XXIV is primarily expressed in bone and cornea, both of which contain heterotypic fibrils composed of collagens I and V, while collagen XXVII is strongly associated with cartilage [96]. These findings suggest that these collagens play specific roles in the development and maintenance of these tissues. Additionally, the identification of Fibril-Associated Collagens with Interrupted Triple Helices (FACITs) has introduced a subclass of collagens that interact with fibrils to form more complex, higher-order structures [84]. FACITs, including collagens IX, XII, XIV, and XVI, are characterized by interruptions in their triple helix domains, enabling them to interact with other ECM components and influence the biomechanical properties of tissues [84, 97]. These collagens are implicated in various processes, including tissue development, wound healing, and matrix stabilization [84, 97].

Collagen’s biocompatibility, low toxicity, and favorable chemical and immunological properties make it a highly versatile biomaterial in the field of tissue engineering and regenerative medicine [98]. Collagen type I, in particular, is widely used due to its ability to form hydrogels upon changes in pH, which can be processed into various forms, including sponges, sheets, and scaffolds. These properties make collagen ideal for creating scaffolds that support cell growth and tissue regeneration, particularly in bone, nerve, and vascular repair [98, 99]. Additionally, collagen-based biomaterials can be sourced from various animal tissues, such as bovine tendon, porcine skin, and rat tail, which are commonly used in medical applications like wound dressings and skin substitutes [100, 101]. The degradability of collagen by matrix metalloproteinases (MMPs) further enhances its utility in tissue engineering, as it allows for the natural remodelling and integration of collagen-based scaffolds into the host tissue [101, 102]. Collagen is also used in cosmetic applications, drug delivery systems, and as a platform for cell culture, underscoring its versatility in both clinical and research settings [98].

Membrane collagens, such as collagens XIII, XVII, XXIII, and XXV, play significant roles in cellular processes beyond structural support. These collagens are type II transmembrane proteins that extend into the extracellular space, where they interact with other ECM components and contribute to processes like cell adhesion, signalling, and wound healing [84]. For instance, collagen

XIII is involved in cell-matrix and cell-cell interactions, which are critical for tissue integrity and repair. Collagen XVII, also known as BP180, is a key component of hemidesmosomes in epithelial cells, where it interacts with other proteins to anchor the epidermis to the underlying basement membrane. Collagen XXV, also known as CLAC-P (Collagen-Like Alzheimer amyloid plaque Component Precursor), is particularly important in neurodegenerative diseases like Alzheimer's, where it is deposited in beta-amyloid plaques [84]. This suggests that collagen XXV plays a role in the formation of these plaques and in the associated neuronal degeneration, highlighting the diverse functions of collagen in both health and disease.

1.6.2 Fibronectin

Fibronectin, an ECM glycoprotein, plays a crucial role in development and tissue formation [103]. It exists in two forms: a soluble plasma form, which circulates in the blood, and an insoluble fibrillar form found in the ECM [104]. The soluble plasma form of fibronectin is produced by hepatocytes and is present at high concentrations in plasma [103]. Meanwhile, soluble cellular fibronectin is produced by a variety of cells, including mesenchymal cells, fibroblasts, smooth muscle cells, epithelial cells, and endothelial cells [103]. The fibronectin gene is located on human chromosome 2 [103, 105] and on chromosome 1 in mice [103, 106]. Despite being a product of a single gene, fibronectin can exist in up to twenty different isoforms, due to alternative splicing of its pre-mRNA [107].

Fibronectin is a prominent component of many tissues throughout the body. It has been detected in various bodily fluids, including plasma, amniotic fluid, bronchoalveolar fluid, and synovial fluid [108]. Fibrillar fibronectin is found in a wide range of tissues such as lymphatic tissue, blood vessels, muscle, the respiratory tract, glands, liver, gastrointestinal tract, kidney, skin, and connective tissue [109]. Fibronectin is particularly abundant around smooth muscle cells and striated muscle fibers, within the connective tissue stroma of lymph nodes and the spleen, and in the sinusoidal walls of the liver [109]. However, its presence is limited in mature cartilage and the nervous system [103]. The widespread distribution of fibronectin throughout the body underscores its critical role in maintaining tissue homeostasis.

Fibronectin Structure

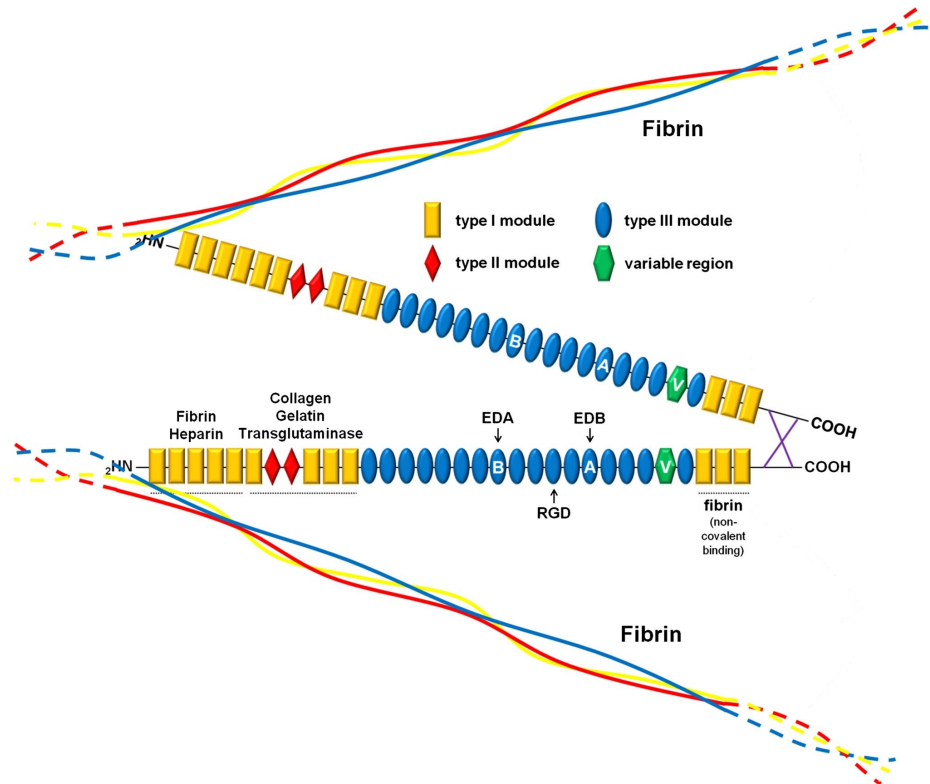


Figure 1.4: Fibronectin structure. Structure of fibronectin. Two alternative splicing sites exist within type III modules, called EDA and EDB. Dotted lines represent the binding sites of various ligands. While cFn contains EDA, EDB, or both, pFn lacks EDA and EDB. The N-terminal 27 kDa region of fibronectin can be covalently linked with fibrin. This interaction may enhance lateral aggregation of fibrin fibers. The fibronectin–fibrin complex formed at the site of vascular injury also provides multiple binding sites for platelets and other cells in the circulation[110]

Soluble fibronectin is a dimeric glycoprotein consisting of two almost identical monomers linked at their carboxy-terminus by two interchain disulfide bonds (Figure 1.4) [103]. Each monomer weighs approximately 250 kD and is composed of three types of repeating homologous subunits: type I (FNI), type II (FNII), and type III (FNIII) [103]. Each monomer contains twelve type I, two type II, and fifteen to seventeen type III subunits [103, 110].

Variations in the number of type III subunits occur due to alternative splicing [107]. Exon skipping or usage leads to the inclusion or exclusion of one or two extra type III domains (ED-A, ED-B) [107, 110]. The ED-A and ED-B domains are present only in cellular fibronectin [103]. Nine of the twelve type I subunits are located in the amino-terminal region of fibronectin, while the remaining type I subunits are situated at the carboxy-terminus [107]. The type II subunits are located between the sixth and seventh type I subunits [107], and the type III subunits are found between the first and tenth type I subunits [107]. The type I and II subunits are approximately 40 and 60 amino acids long, respectively, and are stabilized by two disulfide bonds that link their beta strands [107]. Type III subunits are about 90 amino acids long [107] and consist of seven β -strands arranged in anti-parallel β -sheets connected by flexible loops [111]. Unlike type I and II subunits, type III subunits do not contain disulfide bonds, which gives them a labile structure capable of unfolding and refolding in response to applied forces [112, 113].

Fibronectin Binding Sites

Cell Surface Receptors Fibronectin interacts with several cell surface receptors, including heparan sulfate proteoglycans and integrins [103, 114]. Heparan sulfate proteoglycans are ECM proteins characterized by their glycosaminoglycan chains covalently linked to a core protein [115]. These proteoglycans exist in both ECM-bound and membrane-bound forms [115]. Fibronectin has at least three heparin-binding domains that mediate its cellular effects.

Fibronectin-integrin interactions are vital for regulating several cellular behaviours, including migration [116, 117], vascular development [118], and cell cohesion [119]. Fibronectin can bind a variety of integrins, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_V\beta_3$, $\alpha_4\beta_7$, and $\alpha_5\beta_1$ integrins [120]. However, the $\alpha_5\beta_1$ integrin is the primary receptor for fibronectin [107]. The RGD sequence found in FNIII10 mediates integrin binding [107]. RGD peptides can block integrin-mediated cell adhesion to fibronectin [103]. Additionally, the PHSRN sequence in FNIII9 stabilizes the $\alpha_5\beta_1$ integrin-specific binding of the RGD sequence in FNIII10 [121]. Mutations in the synergy site can cause fibronectin binding to shift from $\alpha_5\beta_1$ to $\alpha_V\beta_3$ integrins [117]. Furthermore, FNIII14-V can also bind integrins, primarily recognizing α_4 -containing integrins [120]. The ability of fibronectin to engage with multiple integrins may significantly influence the cellular response to fibronectin.

ECM Binding Sites Fibronectin has several ECM ligand-binding sites essential for its functions [107], including binding sites for collagen, heparan sulfate proteoglycans, and fibrin. Fibronectin interacts with collagen and denatured collagen (gelatin) through the FNII1-2 and FNII7-9 subunits [122, 123, 124]. Additionally, this binding is supported by hydrophobic interactions with collagen, which help strengthen fibronectin's attachment within the ECM [125]. Interestingly, the collagen triple helix may locally unfold under normal physiological conditions [126], suggesting that fibronectin's ability to bind gelatin in vitro may also hold physiological relevance. This unfolding could allow fibronectin to play a natural role in processes like tissue repair or remodelling by enabling closer interactions with partially unfolded collagen.

Fibronectin can interact with various collagen types, including type I, II, and III [22, 125]. These interactions affect cell adhesion, influencing cell morphology and are necessary for fibronectin-directed collagen I deposition [127]. Fibronectin-induced collagen deposition further modulates cellular responses to fibronectin [127], highlighting the importance of fibronectin-collagen interactions in ECM composition and cell function regulation.

Fibronectin also binds to heparan sulfate proteoglycans through several heparin-binding subunits [128]. The molecule has at least four heparin-binding subunits: FNII1-5 [128, 129], FNIII1 [40, 128], FNIII5 [130], and FNIII13 [131].

Fibroblasts attach to fibronectin fragments containing the central cell-binding domain but fail to spread when the FNIII13 domain is absent [114, 132]. The heparin-binding domain is critical for maximal motility in several cell types [133], while FNIII5 and FNIII13 domains block fibronectin matrix assembly [60, 130]. This site is cryptic in soluble fibronectin but becomes exposed in ECM fibronectin, where it can induce proliferation, migration, and contractility [40, 134, 135].

In vivo, this fragment induces vasodilation [44]. These findings suggest that fibronectin's interaction with heparan sulfate proteoglycans plays a key role in matrix assembly and mediating cellular effects. Additionally, fibronectin contains two major fibrin-binding sites located in the amino-terminal (FNII1-5) and carboxy-terminal (FNII10-12) regions [136]. Cell attachment and migration on fibrin clots require fibronectin [137, 138, 139], underscoring the importance of fibronectin-fibrin interactions in wound healing.

Self-Association Sites Fibronectin contains multiple self-association sites [140], which are important for its matrix assembly [140]. The first type III repeat, FNIII1, is a critical self-association site that interacts with several distinct fibronectin sites, including FNII1-5, FNIII5, and FNIII7 [141]. FNIII1 can bind conformationally altered FNIII10 in an RGD-independent manner [142]. Moreover, a 31-amino-acid fragment from FNIII1 can promote fibronectin self-association [143]. Additional self-association sites are accessible in soluble fibronectin and are essential for fibrillogenesis, including FNIII2, FNIII3, and FNIII11 [144, 145]. These sites help regulate fibronectin's transition from

its soluble form to an ECM form.

Fibronectin Matrix Assembly Incorporation of soluble fibronectin into ECM fibronectin fibrils occurs through a coordinated process known as fibronectin matrix assembly [146]. Fibronectin matrix assembly is a cell-driven, stepwise process beginning with soluble Fn binding to the cell surface, primarily through the N-terminal 70-kDa region. This initial binding is saturable and reversible. $\alpha 5 \beta 1$ integrins are the primary integrins involved in fibronectin matrix assembly. Once bound, Fn interacts with other Fn molecules, forming disulfide-stabilized multimers. The cell surface then regenerates binding sites, allowing further Fn attachment at specialized sites where assembly occurs.

Both integrins and large cell surface molecules known as LAMMs are essential for organizing Fn into a structured matrix. Cells like fibroblasts, endothelial cells, and vascular smooth muscle cells play key roles by secreting Fn and assembling it into the ECM. Fn incorporated into the ECM can come from plasma or be synthesized locally by cells, ensuring a robust and dynamic Fn network [146]. The ECM form of fibronectin is responsible for the majority of fibronectin's effects on cell function [147, 148]. Fibronectin self-association is critical for fibril formation, with FNIII1 containing self-association sites that become exposed upon conformational changes [143]. The $\alpha 5 \beta 1$ integrin applies tensile force to fibronectin, inducing fibril elongation [149]. Actin-myosin-mediated cytoskeletal contractility enhances fibronectin matrix assembly [104], while inhibitors of contractility reduce matrix assembly [104].

Fibronectin Function

Fibronectin is essential for development, as demonstrated by genetic studies in mice, where inactivation of the fibronectin gene results in early gestational death and severe developmental defects [47, 52, 46, 150]. In *Xenopus*, knockdown of fibronectin or $\alpha 5 \beta 1$ integrins disrupts convergent extension, further emphasizing the critical role of fibronectin in tissue formation [151].

In vitro models demonstrate fibronectin's role in tissue formation. Fibronectin regulates epithelial cell proliferation in acinar structures of the mammary gland [152]. Fibronectin also promotes 3D tissue-like layers in fibroblasts [153], while supporting vasculogenesis in endothelial cells embedded in 3D fibrin matrices [154]. These studies collectively highlight fibronectin's crucial role in tissue development [150].

ECM fibronectin has been shown to influence several cellular behaviours, including increased proliferation, migration, and contractility, as well as enhanced tensile strength in collagen-based tissues as we explained earlier [18, 21, 38, 39, 22, 152]. Fibronectin modulates the deposition of ECM proteins such as collagen types I and III, thrombospondin, and fibrillin [155]. However, the mechanisms by which ECM fibronectin exerts different effects compared to its soluble form are not entirely clear. Studies using engineered fusion proteins have shown that the heparin-binding site in FNIII1 mediates ECM fibronectin effects on cell behaviour, including spreading, growth, contractility, and migration [135]. Peptides containing the heparin-binding region of FNIII1 inhibit fibronectin-induced cell proliferation [135], providing further evidence that ECM fibronectin exerts unique effects on cellular processes through specific interactions with exposed cryptic sites.

1.7 Scope and Objectives of This Thesis

The preceding review has highlighted the essential role of the ECM in regulating cell behaviour, providing structural support, and orchestrating dynamic processes such as adhesion, migration, and mechanotransduction. Among the wide array of ECM components, collagen and fibronectin have emerged as two of the most influential proteins, due to their structural dominance and their interactions with integrins and other cell surface receptors. Furthermore, the ECM is not a static structure, it undergoes continuous remodelling in response to cellular activity, and its mechanical and biochemical characteristics are tightly linked to tissue function and healing.

Given this complexity, there remains a need to develop in vitro systems that better mimic the structural and mechanical properties of native ECM environments, particularly those capable of

capturing how cells respond to, and reshape, their surroundings. While collagen-based scaffolds are widely used for this purpose, their performance can be significantly enhanced by incorporating additional ECM proteins such as fibronectin, and by controlling fabrication parameters that affect scaffold architecture and mechanical behaviour.

This thesis addresses these needs by focusing on the design and characterization of three-dimensional collagen-fibronectin (ColFn) scaffolds. The scope of the research includes:

- Developing a temperature-controlled method to fabricate tunable ColFn hydrogels (Chapter 3);
- Investigating the role of fibronectin in scaffold organization, stability, and cell adhesion (Chapters 4 and 5);
- Exploring how fibroblast cells, at varying densities, reorganize the ECM and influence its mechanical properties (Chapter 6).

The overall objective is to understand how scaffold composition and cellular interactions influence the physical behaviour of engineered ECM systems. By combining scaffold design with the study of cell-driven reorganization, this work aims to contribute to the development of biomaterials that more faithfully replicate tissue environments and support regenerative outcomes.

Through this integrated approach, the thesis provides a framework to better understand the interplay between ECM composition, scaffold fabrication conditions, and cellular behaviour. By systematically analyzing both acellular and cell-laden systems, the study offers insights into how fibronectin contributes to scaffold architecture and stability, and how fibroblasts dynamically reorganize their microenvironment in response to physical and biochemical cues. These findings are expected to enhance the design of biologically responsive scaffolds, inform strategies for controlling cell behaviour through material engineering, and support future applications in tissue regeneration, wound healing, and the modeling of cell-matrix interactions under physiologically relevant conditions. Together, these studies not only deepen our understanding of ECM-cell interactions but also provide actionable insights for designing next-generation biomaterials that emulate physiological behaviour and promote tissue regeneration.

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Chapter 2

Experimental Techniques and Instrumentation

2.1 Introduction

Soft, fibrillar hydrogels composed of type I collagen and fibronectin are extensively utilized as ECM analogues due to their ability to replicate critical properties such as porosity, ligand chemistry, and mechanical behaviour inherent to native connective tissues [1]. These matrices are biologically significant because of their high water content, intricate internal architectures, and dynamic responsiveness to cellular remodeling. However, these attributes simultaneously introduce complexities into their biophysical characterization. A single analytical technique is insufficient to concurrently resolve the network architecture at the microscale, quantify bulk viscoelastic properties, and monitor real-time cellular interactions within the matrix. Therefore, this thesis integrates oscillatory shear rheology, which probes scaffold mechanics, with confocal laser-scanning microscopy (CLSM), which resolves microstructural and cellular organization, to provide a comprehensive understanding of structure–function relationships in fibroblast-seeded collagen–fibronectin hydrogels.

Rotational rheometry serves as the gold-standard for quantifying the storage (G') and loss (G'') moduli, which characterize how hydrogels resist deformation and dissipate mechanical stress. Frequency and strain sweep experiments provide critical insights into not only linear viscoelastic parameters but also into nonlinear phenomena such as strain-stiffening and stress-relaxation, both essential characteristics of load-bearing soft tissues [2]. Additionally, bulk rheology offers a time-resolved analysis of factors including polymer concentration, fibrillogenesis kinetics controlled via temperature, and cell-mediated matrix compaction.

Confocal laser-scanning microscopy complements rheology by enabling non-destructive, high-resolution imaging of fully hydrated, three-dimensional networks with sub-micron axial precision, a capability unattainable with traditional electron microscopy due to dehydration, induced collapse [3]. Operating in reflection mode leverages the intrinsic refractive index contrast between collagen fibrils and surrounding aqueous medium, eliminating the need for external labeling. Simultaneously, fluorescence or second-harmonic generation modalities can specifically highlight components such as fibronectin, labeled collagen molecules, or live fibroblasts without perturbing the hydrogel’s structural integrity [4]. This imaging methodology provides quantitative measures of fibre diameter, pore size, alignment, and cellular traction events, microstructural parameters intimately linked to the bulk viscoelastic properties measured rheologically.

When rheology and CLSM are applied together, whether sequentially or via integrated “rheo-confocal” instrumentation, they enable direct correlations between structural and mechanical data. Landmark studies in confocal rheology demonstrated that the establishment of an interconnected collagen network visually correlates with the characteristic G'/G'' crossover point indicative of gelation [5, 6]. Currently available commercial systems have streamlined this multimodal approach, facilitating real-time observations of microstructural dynamics under defined mechanical stimuli

such as shear or compression. By incorporating live-cell fluorescence imaging, the present work extends this capability, providing critical insights into how fibroblast behaviours, dynamically influence viscoelastic properties. While rheology and CLSM are often integrated into simultaneous ‘rheo-confocal’ systems for real-time correlation of mechanical and structural data, this thesis employed the two methods in a sequential but complementary manner.

While alternative methods such as atomic force microscopy indentation, Brillouin microscopy, dynamic light scattering, and bulk turbidimetry have been frequently employed to study hydrogels, these techniques typically focus on limited length-scales or involve invasive sample preparation. In contrast, the integrated rheology–CLSM approach described here spans scales from nanometers to millimeters, maintains sample hydration, remains non-destructive, and supports live-cell experiments, features indispensable for the in situ investigation of cell-mediated matrix remodeling. Consequently, this dual modality has emerged as a standard workflow in soft-matter and tissue-engineering laboratories studying collagenous and other biopolymeric hydrogel systems [5].

The remainder of this chapter proceeds in two structured sections. First, detailed theory, rheological methods and their relevance to mechanistic questions are presented. Second, the confocal imaging protocols, encompassing reflection, fluorescence, and time-lapse imaging techniques, are systematically described to elucidate the hydrogel’s fibre architecture and cellular interactions. Collectively, these complementary techniques provide the quantitative foundation necessary for subsequent chapters, where structure–mechanics–cell behaviour relationships elucidate emergent phenomena such as fibroblast-induced stiffening and mesh-dependent viscoelastic responses within collagen–fibronectin hydrogels.

2.2 Confocal Microscopy for Hydrogel Characterization

2.2.1 Principles of Confocal Imaging

CLSM is an advanced optical imaging technique that produces high-resolution, optically sectioned images by rejecting out-of-focus light. In a confocal microscope, a focused laser beam scans across the sample and a pinhole aperture in front of the detector blocks out-of-focus fluorescence or reflected light, so only signal from the focal plane is collected [7]. By scanning in a raster pattern and detecting point-by-point, the confocal yields a thin optical slice of the specimen at a specific depth. Stacking multiple optical sections along the vertical (z) axis allows reconstruction of a sharp three-dimensional image of the sample [3]. Figure 2.1 illustrates the basic layout of a CLSM system, including the objective lens focusing the laser into the sample, dichroic mirrors to separate excitation and emission light, and the pinhole and photomultiplier detector that enable optical sectioning. The result is a dramatic improvement in image clarity for thick specimens compared to conventional widefield microscopy.

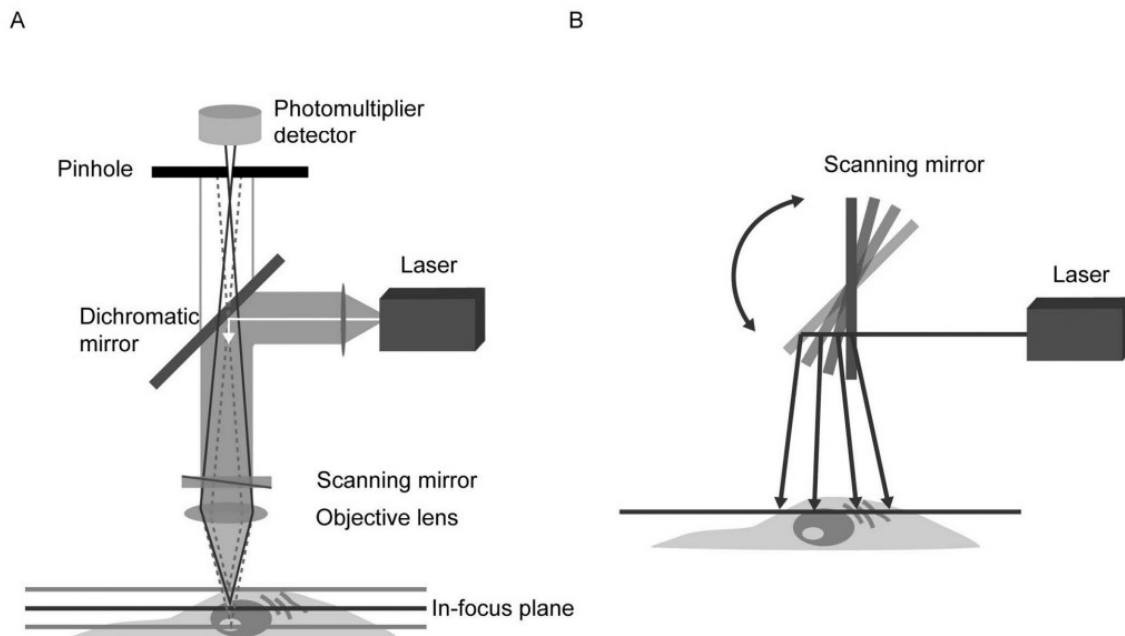


Figure 2.1: Simplified schematic of a confocal laser scanning microscope. (A) Light from a laser is focused by the objective into a small spot in the sample (*in-focus plane*). Emitted or reflected light from that focal plane is descanned and passes through a pinhole aperture conjugate to the focal plane, which blocks out-of-focus light (dashed rays). A photomultiplier detector then registers the *in-focus* signal. (B) A fast scanning mirror is used to raster the laser point across the sample in *X-Y*, building up an image pixel by pixel. By acquiring images at successive focal depths (*Z*), a 3D dataset can be obtained [7].

In widefield microscopes, a thick or multilayered sample produces a blurred image because out-of-focus regions contribute background haze; at high magnification, only a $\sim 1 \mu\text{m}$ -thick plane is truly in focus, and everything above and below it obscures details. Confocal microscopy avoids this by capturing only in-focus signal, yielding crisp images even in samples thicker than a few cell layers.

The ability to optically section and reconstruct 3D volumes makes CLSM especially well-suited for studies of ECM-mimicking hydrogels, which are typically thick, hydrated, and turbid. Unlike electron microscopy, which can achieve nanometer resolution but requires extensive sample processing (fixation, dehydration, embedding, or coating) and thin physical sectioning, confocal imaging can be performed in situ on intact, fully hydrated gels [3, 8]. No drying or slicing is needed because the microscope’s optical sectioning captures internal slices noninvasively, preserving the hydrogel’s native swollen structure.

This is critical for ECM-like collagen–fibronectin hydrogels, which can contain $> 90\%$ water – drying such a sample for SEM or TEM would collapse the porous network and alter fiber architecture, obscuring the native morphology and mechanics [3]. Confocal microscopy, by contrast, allows visualization of the internal microstructure of the hydrogel in its natural, hydrated state and even during dynamic processes. Indeed, the preparation-free and nondestructive nature of fluorescence/confocal imaging permits in situ, real-time observation of 3D hydrogel networks with high contrast [3]. The achievable spatial resolution (approximately 200 nm lateral and 600–800 nm axial with high numerical aperture lenses [7]) is sufficient to resolve key structural features of ECM-mimicking hydrogels, such as cell bodies ($\sim 10\text{--}50 \mu\text{m}$), cell protrusions, and fibrous collagen strands on the order of a micron in diameter [7]. While confocal resolution is lower than that of electron microscopy, it strikes a practical balance; it can visualize micron-scale fiber bundles and cell–matrix interactions which govern mechanical and biological behaviour, without sacrificing the sample’s viability or 3D integrity. Another major advantage of CLSM for ECM studies is the ability to image thick, optically dense samples by collecting serial optical sections. Collagen-rich hydrogels are often millimeters in size, far beyond the depth that traditional microscopes can clearly image. Confocal’s optical sectioning,

combined with the use of appropriate objectives and image reconstruction, enables examination of such thick specimens. Optical sectioning avoids the need to physically section the gel, thereby preventing introduction of artifacts and allowing one to image the same sample volume repeatedly over time. This is crucial for ECM studies where one might want to monitor how cells remodel their surrounding matrix or how the network structure evolves (for instance, fiber alignment or crosslinking changes) under various conditions. Confocal time-lapse imaging can capture these dynamics.

The confocal microscope’s ability to provide optical slicing, 3D imaging, and multi-channel fluorescence in live, hydrated environments makes it ideally suited for visualizing extracellular matrix-like hydrogels and their interaction with embedded cells [3]. Researchers have noted that confocal techniques precisely visualize the 3D microstructure of hydrogel networks and even enable live monitoring of structural changes and molecular transport in real time [3]. These capabilities are central to our characterization of collagen–fibronectin gels with fibroblasts, as they allow us not only to capture static snapshots of the matrix architecture but also to observe how cells and the matrix influence each other throughout the experiment.

2.2.2 Imaging Modalities Used

In this thesis, we employed multiple confocal imaging modalities to fully characterize the cell-laden collagen–fibronectin hydrogels. Reflection mode was used to visualize the collagenous fiber network, and fluorescence mode was used to visualize the seeded fibroblasts, specific cellular structures, and fibronectin fibers. Each modality provided complementary information about the 3D culture system.

Confocal reflection imaging was used to capture the collagen fiber architecture in the hydrogels without any labeling. In reflectance mode, the microscope detects light that is backscattered or reflected from structures in the sample when the laser is focused on them. Fibrillar collagen is particularly suitable for reflection microscopy because its fibers have a different refractive index than the surrounding water and are roughly the same size as the illumination wavelength. This causes them to reflect and scatter a portion of the incident laser light. By placing a detector at the excitation laser wavelength (using a suitable dichroic mirror), the confocal system collects this backscattered light as an intrinsic signal. The result is a label-free image of the fibers, with collagen bundles appearing as bright linear structures against a dark background. This approach eliminates the need for staining, and the optical sectioning capability removes the requirement for physical thin-sectioning, thus minimizing processing and preserving the natural structure of the collagen network [8].

We excited the sample with a 488 nm laser and captured the reflected light through the same objective, producing a high-contrast monochromatic image of the fibrous matrix. Reflection mode, detailed earlier, was used here to map collagen fiber organization with micron-scale resolution, as demonstrated in prior studies [8]. In our hydrogels, the reflection images similarly reveal the mesh of collagen fibers, showing details such as fiber thickness, entangled branch points, and pores between fibers. This mode is especially powerful because it is entirely nondestructive – the same sample can be imaged repeatedly over time to see how the fiber network evolves as cells exert forces or as the gel matures. Indeed, confocal reflection microscopy has been called a physical approach for monitoring ECM structure, cell–matrix interactions and changes in ECM structure over time in 3D cultures [9]. By using reflection imaging, we could visualize how fibroblasts rearrange or compact collagen fibers in real time, without needing any fluorescent labels in the matrix. Alongside reflection, we used confocal fluorescence imaging to visualize the fibronectin fibers and the fibroblast cells within the hydrogel.

The fibronectin in our collagen–fibronectin hydrogel scaffolds was fluorescently labeled with Alexa Fluor 647, enabling its visualization in the far-red channel. Upon excitation with the 633 nm laser, the labeled fibronectin emitted fluorescence with a peak around ~668 nm, which was detected using the confocal microscope’s far-red emission detector. This fluorescent labeling facilitated the observation of fibronectin fiber distribution and morphology within the 3D hydrogel network, even in the presence of GFP-expressing fibroblasts. By clearly distinguishing between cellular (blue) and matrix (far-red) components, we were able to monitor cell–matrix interactions and remodeling processes in situ with high spatial resolution.

The fibroblasts in our model were genetically labeled with green fluorescent protein (GFP), which caused them to fluoresce in the green channel. Upon excitation with the 488 nm laser, the GFP-expressing fibroblasts emitted green light (peak ~ 509 nm) that was captured by the confocal’s green emission detector. The whole-cell GFP provided a general view of the cell bodies and processes, as the protein was expressed throughout the cytoplasm and nucleus, outlining the overall cell shape.

All fluorescence imaging was conducted with appropriate channel settings to avoid crosstalk between signals. We used sequential scanning for each fluorescent channel and the reflection channel. For example, the 488 nm laser excitation was used for both GFP and reflection imaging (with detection in separate bandwidths), while the 633 nm laser was used for detecting fibronectin fibers, each captured on separate detectors. By carefully selecting filter sets and scan sequences, we prevented strong reflection or GFP signals from bleeding into the far-red channel, and vice versa. This approach produced clear multi-channel images, where collagen fibers, fibronectin fibers, and cell shapes could be visualized together in 3D.

Furthermore, a key capability of our confocal approach was time-lapse imaging of live cell–matrix interactions. We conducted live imaging experiments and acquired images repeatedly over several hours. During these experiments, the fibroblasts remained viable and could be observed migrating and remodeling the surrounding collagen and fibronectin. The confocal was operated in reflection mode (to monitor fiber movements) and fluorescence mode (to track the GFP-labeled NIH/3T3 cells) concurrently over time. Because confocal imaging is relatively fast and can be gentle when settings are optimized, we were able to capture a series of 3D z-stacks at set intervals, effectively creating a 4D dataset (3D structure over time). This allowed us to track cell activity and matrix remodeling dynamically, for example, observing a fibroblast pulling on a collagen fiber to reorient it, or a cluster of cells contracting the gel and increasing local fiber density. Such live imaging is a major advantage of CLSM over fixed imaging methods: in situ time-lapse confocal imaging provides real-time visualization of structural changes and matter transport within hydrogels [3]. In our case, it provided direct evidence of how fibroblasts mechanically remodel collagen–fibronectin matrices.

2.2.3 Optical Considerations in 3D Hydrogels

Imaging thick, highly hydrated 3D hydrogels by confocal microscopy required careful consideration of optical limitations and the implementation of strategies to overcome these challenges. The primary issues we addressed were limited depth penetration and photobleaching.

Depth penetration and dual-side imaging

In confocal microscopy, as the focal plane moves deeper into a sample, the signal typically becomes attenuated and distorted. This is due to light scattering and absorption within the specimen, as well as reduced detection efficiency for light originating from deeper layers. In a thick collagen gel, which can be opaque over millimeter distances, the intensity of fluorescence or reflected light often decreases markedly with depth. This means that deeper sections may appear dimmer and lower in contrast, even if the underlying structure is unchanged, because the intervening material scatters both the excitation and emission light [10]. Additionally, beyond a certain depth, the confocal’s axial resolution and ability to reject out-of-focus light degrade, resulting in image blurring. In practice, a standard one-photon confocal microscope can typically image up to about 100–200 μm into a dense tissue or gel before image quality significantly deteriorates, though this depends on the optical properties of the sample and the objective lens used. Our collagen–fibronectin hydrogels, however, were approximately ~ 1.6 mm thick, far exceeding this depth limit. To successfully image the entire volume, we implemented a dual-side z-stack imaging approach.

This approach involved acquiring confocal z-stacks from both the top and bottom of the gel, then merging them to cover the full thickness. In practice, we focused the microscope into the sample from the top surface and collected optical sections down to the depth limit where the signal became too weak. Afterward, we inverted the sample or used an inverted microscope to image from the bottom side upward, collecting optical sections from the opposite face to cover additional depth until the two stacks overlapped. This dual-view imaging technique is conceptually similar to

the multi-view approach used in light sheet microscopy for thick specimens [11]. It enabled us to obtain a complete 3D dataset of thick hydrogels without the need for physical sectioning or chemical clearing. The two half-stacks were later computationally stitched at the mid-plane to reconstruct the full volume. Although some signal loss remained at the extreme center, as this region was furthest from either imaging side, the dual-side approach significantly improved the uniformity of illumination and preserved detail throughout the depth of the sample.

Photobleaching

When performing confocal microscopy on live samples, it is essential to manage photobleaching of fluorophores. High-intensity laser illumination can rapidly bleach fluorescent proteins and dyes, and it can also generate reactive oxygen species that damage live cells. This concern was even more critical for our experiments, as 3D time-lapse imaging requires repeated exposure of the same sample. We adopted several strategies to minimize photobleaching. First, we optimized the laser power to be as low as possible while still achieving a good signal-to-noise ratio. Instead of using the maximum laser output, we calibrated the system to ensure that the brightest features of interest were just adequately illuminated. In practice, this involved using neutral density filters and setting laser power to low levels, such as just a few percent of the 488 nm laser for GFP imaging, while relying on the sensitivity of the detector gain to amplify the signal. By keeping the light intensity low, we significantly reduced photobleaching per image.

We also limited the exposure time by using faster scanning speeds and fewer line averages. Confocal scanners allow adjustment between scanning speed and image quality, where slower scans improve signal quality but also increase the total light dose. We selected a scan speed that provided acceptable image clarity with minimal averaging, thereby reducing the time the laser dwelled on each pixel. This approach is supported by the principle that faster scanning reduces photobleaching, even though it can introduce slightly more image noise. We identified an optimal balance where the images remained clear while photobleaching was significantly reduced compared to slower scans.

Additionally, for time-lapse experiments, we often restricted both the imaging volume and the imaging frequency. Instead of scanning the entire gel at regular intervals, we focused on smaller regions of interest and acquired optical sections at coarser z-intervals. This strategy minimized the exposure of each cell or location to laser light during the course of the experiment, ensuring that the cells remained viable and behaved normally throughout imaging.

An important advantage of our setup was that reflection mode imaging did not suffer from photobleaching. Since it relies on scattered light rather than fluorophores, it allowed us to image the collagen network repeatedly without any fading. This capability enabled us to monitor the matrix structure extensively during time-lapse experiments. Only the fluorescent channels used for imaging the NIH fibroblasts and fibronectin fibers were susceptible to bleaching.

We also took advantage of the high sensitivity of our confocal detectors. By using high detector gain and adjusting the offset appropriately, we were able to lower the laser power while still obtaining usable images. We calibrated the gain settings to provide good signal from GFP at low laser power, accepting slightly higher image noise, which could be mitigated during post-processing if needed.

2.2.4 Imaging Parameters and Standardization

To ensure reliable and comparable results across all our confocal imaging sessions, we rigorously standardized the key imaging parameters. Consistent settings are crucial for quantitative image analysis, since varying parameters like laser power or detector gain between samples could introduce bias. We first determined the optimal imaging settings on trial samples and then kept these parameters constant for all experimental runs.

The main confocal settings we standardized were pinhole size, laser power (intensity), detector gain, and offset. Each of these settings affects image quality.

Pinhole Size

We set the confocal pinhole to approximately one Airy Unit for each channel, which corresponds to an optical section thickness equal to the microscope’s depth of focus at the emission wavelength. A one Airy Unit pinhole is a standard choice because it maximizes optical sectioning by rejecting out-of-focus light while collecting most of the in-focus signal. Using a significantly larger pinhole would allow more light to enter, producing a brighter image but reducing z-resolution and introducing more background blur. Conversely, using a much smaller pinhole would slightly improve axial resolution but drastically reduce image brightness.

Laser Power

We carefully adjusted the laser power for each channel during the initial setup so that the brightest features in the sample produced an adequate signal without saturation. Once determined, this laser power setting was fixed and used for all samples in that experimental series. This consistency was essential because light intensity directly affects image brightness. To compare fluorescence levels or image-derived metrics between samples, the illumination had to remain constant.

Detector Gain and Offset

The confocal’s photomultiplier tube (PMT) detectors have adjustable gain and offset settings. We optimized the gain so that the image utilized the full dynamic range of the detector without clipping, while the offset was set so that background areas registered zero intensity, avoiding a raised black level. Once we identified a gain and offset combination that provided good contrast for a representative sample, we kept those settings fixed for all subsequent images in that channel. Maintaining consistency in gain and offset was critical because a sample imaged with a higher gain would appear artificially brighter, which could be misinterpreted as increased fluorescence or structural differences such as thicker fibers. By keeping the gain constant, any differences in image intensity reflected true differences in labeling or structure rather than changes in detector sensitivity. This practice aligns with established guidelines recommending identical acquisition settings for all specimens to ensure valid comparisons of intensity or morphology.

By maintaining consistent imaging conditions, we improved the reliability of quantitative comparisons. For example, if one hydrogel contained slightly thicker fibers than another, that difference would be consistently reflected under the same imaging settings. This approach avoided misleading interpretations, such as mistaking a brighter image for greater fiber density when it could be due to a higher detector sensitivity on a given day. This standardization aligns with best practices for quantitative fluorescence imaging, which emphasize that acquisition settings must remain constant between specimen groups for valid intensity and structural comparisons.

2.2.5 Quantitative Image Analysis

The rich 3D datasets obtained from confocal imaging were analyzed quantitatively using a combination of the Zeiss ZEN Blue software for initial processing and colocalization analysis, and ImageJ/Fiji for detailed image analysis with various plugins. Our goal was to extract meaningful structural and biological metrics from the images, such as fiber architecture parameters and cell morphology descriptors, and to correlate these with the hydrogel’s mechanical or biological performance.

From the confocal images of the collagen–fibronectin matrix, we quantified several aspects of the fibrous network. We measured the thickness, length, and pore area of collagen fibers as they appeared in the reflection images. This analysis involved first calibrating the image scale (micrometers per pixel) and then manually measuring representative fibers. The typical collagen fiber diameters we observed ranged from approximately 1 to 5 μm , and the lengths were about 10 to 70 μm [8]. Since confocal reflection imaging is sensitive to structures near the focal plane, it reliably resolves fibers within this size range. Prior studies have used confocal reflection to measure collagen fiber diameters, demonstrating that this method provides measurements comparable to electron microscopy for fibers in the micron range [12].

2.2.6 Advantages Over Other Imaging Modalities

In the context of hydrated, cell-populated collagen–fibronectin hydrogels, CLSM provided an optimal balance of resolution, depth, and live imaging capability. We were able to image the intact three-dimensional structure of the gel without disturbing it, observe living cells interacting with the matrix in real time, and capture multiple channels of information in a single dataset. While electron microscopy offers higher raw resolution, it cannot capture dynamic or three-dimensional aspects and requires destroying the sample’s native state. Thus, confocal microscopy was invaluable for this thesis, enabling us to characterize both the microstructural organization of the ECM-like hydrogel and the behaviour of cells within it in a way no other single technique could achieve. As one review aptly noted, confocal and related fluorescence microscopy techniques enable ultrafast, noninvasive, and high-contrast visualization of hydrogel microstructure, which is key to understanding their physical and biological performance [3]. The extracted structural metrics, fiber diameters, pore sizes, and cell morphology, were correlated with rheological measurements and fibroblast activity, providing integrated insights into scaffold performance. These strengths made CLSM the centerpiece of our hydrogel characterization approach, providing quantitative insights that would have been difficult to obtain using methods such as SEM, TEM, or even non-optical techniques. In conclusion, the use of confocal microscopy significantly advanced our ability to study and interpret the complex three-dimensional interactions between cells and matrix in collagen–fibronectin hydrogels, offering a detailed and dynamic window into the microscale world of engineered ECM.

2.3 Rheology

Rheology, often referred to as the “science of flow and deformation,” originates from the Greek word *rheos*, meaning “stream” or “flow” [13, 14]. Since its inception as a formal discipline in 1929 [13], rheology has expanded to encompass the study of how materials, ranging from fluids to solids, respond to applied forces. This field lies at the intersection of physics, chemistry, engineering, biology, and even medicine, offering a versatile framework for understanding material behaviour under stress. The diverse applications of rheology span from analyzing the mechanical properties of soft tissues to optimizing industrial processes involving polymers, hydrogels, and other complex materials [15].

From a historical perspective, rheology owes much to foundational work by Robert Hooke, who introduced the theory of elasticity in 1678, and Isaac Newton, whose “Principia” in 1687 laid the groundwork for understanding fluid behaviour [13]. These pioneering ideas have since evolved into a sophisticated science that not only characterizes materials but also provides tools for designing solutions to real-world challenges.

At its core, rheology seeks to describe the internal response of materials to forces, bridging the gap between classical theories of elasticity and fluid dynamics [13]. As we explain in the following, elastic solids, governed by Hooke’s law, deform proportionally to the applied stress and recover fully once the stress is removed. Conversely, viscous fluids, as described by Newton’s law, deform continuously under stress, with the rate of deformation proportional to the applied force. Most real-world materials, however, exhibit properties that fall between these two extremes, showcasing viscoelastic behaviour [16]. These materials can deform like elastic solids under certain conditions but also flow like viscous fluids, depending on factors such as stress, time, and temperature [15].

2.3.1 Introduction to Mechanical Properties of Soft Tissues

In the context of polymer science and hydrogel technology, rheology plays a pivotal role in understanding and manipulating material properties for various applications. For instance, the flowability and viscoelastic behaviour of hydrogels are critical in tissue engineering and drug delivery, where precise control over material deformation and flow ensures effective clinical performance [15]. Soft tissues in the human body exhibit diverse and complex mechanical properties due to their composite nature, consisting of collagen and elastic proteins embedded in a hydrated ECM. This ECM, whose

configuration and structural organization vary across tissues, plays a significant role in their functionality and mechanical behaviour. Collagen, in particular, governs muscle elasticity and stiffness, which are influenced by fiber orientation and micromechanical variations [17]. These tissues are often categorized as transversely isotropic due to their anisotropic response to stress, determined by the collagen and elastin fibers in the ECM [17]. Advanced rheological experiments and mathematical models have provided insights into the nonlinear viscoelastic behaviour of tissues, highlighting phenomena such as strain hardening, frequency-dependent properties, and auxetic behaviour. The study of soft tissues, though intricate, is crucial for advancing biomechanics, orthopedics, and regenerative medicine, as it enables a deeper understanding of their physiological and mechanical responses under various loading and deformation conditions [17]. These properties make the study of soft tissues particularly intriguing and essential for applications in biomechanics, orthopedics, and regenerative medicine.

Rheological measurements are conducted using specialized instruments like rheometers, which quantify parameters such as viscosity, elasticity, and gelation time. Techniques like time sweep, strain sweep, and frequency sweep allow researchers to determine critical properties, including the gelation point of hydrogels and the equilibrium modulus plateau [15]. These measurements provide insights into the molecular structure of materials, enabling correlations between material behaviour and their underlying structural organization.

The importance of rheology extends beyond academic interest to practical applications. In industrial settings, rheology guides the design and optimization of processes involving complex fluids such as paints, inks, and polymer melts. For example, the viscoelastic nature of polymer solutions influences their performance during processing and end-use applications [18]. Similarly, in hydrogel fabrication, rheological behaviour affects printability, shape fidelity, and even cell viability during extrusion [15].

Rheology provides a comprehensive framework for studying the deformation and flow of materials, bridging the behaviour of solids and fluids while addressing their intermediate viscoelastic states. This makes it an indispensable tool in understanding the mechanical properties of soft tissues and designing advanced materials for biomedical and industrial applications. As we delve deeper into this field, we will explore specific parameters such as viscosity, elasticity, and viscoelasticity, and discuss how rheometers enable precise measurements of these properties to address contemporary challenges in science and engineering. Given the critical importance of this field, the theory of rheological concepts is presented in the following sections, exploring their foundational principles and applications in greater detail.

2.3.2 Theory of Rheology in Polymeric Materials

In rheology, stress and strain are fundamental parameters used to characterize the mechanical behaviour of hydrogels under deformation. These parameters are indispensable for evaluating the structural integrity and deformation behaviour of hydrogels [19]. When hydrogels are subjected to tensile forces, they undergo elongation in the direction of the applied force. The relative change in length, compared to the original dimension, defines the strain (ε), a dimensionless measure of deformation. The formula for strain is given as:

$$\varepsilon = \frac{\Delta L}{L_0} \quad (2.1)$$

where ΔL is the change in length and L_0 is the initial length.

Stress is the internal resistance of a material to deformation and is calculated as the applied force divided by the area over which the force acts:

$$\sigma = \frac{F}{A} \quad (2.2)$$

where F is the applied force (N) and A is the cross-sectional area (m^2). Stress is measured in Pascals (Pa) and provides a direct indication of the load-bearing capacity of the hydrogel material.

In practical rheological scenarios, hydrogels often experience complex loading conditions where uniaxial assumptions are insufficient. In such cases, stress and strain are represented as tensors to account for multidirectional forces and deformations:

$$\varepsilon_{ij} = \frac{1}{2} \left(\frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right) \quad (2.3)$$

where ε_{ij} is the component of the strain tensor, ∂u_i is the displacement vector component in the i -direction, $\partial x_i \partial x_j$ are spatioial coordinates and finally, $\frac{\partial u_i}{\partial x_j}$ is the gradient of displacement in the j -direction and so forth. $(\varepsilon_{11}, \varepsilon_{22}, \varepsilon_{33})$ are representing elongation or compression along the respective axes and they are called normal strains and $(\varepsilon_{12}, \varepsilon_{13}, \varepsilon_{23})$ describe angular distortions and are called shear strains.

The stress tensor quantifies internal forces distributed over a material's volume, addressing how the material resists deformation. It is written as:

$$\sigma_{ij} = \begin{bmatrix} \sigma_{11} & \sigma_{12} & \sigma_{13} \\ \sigma_{21} & \sigma_{22} & \sigma_{23} \\ \sigma_{31} & \sigma_{32} & \sigma_{33} \end{bmatrix} \quad (2.4)$$

where σ_{ij} is the component of the stress tensor, i, j are directions of the applied force and corresponding surface normal, $(\sigma_{11}, \sigma_{22}, \sigma_{33})$ are representing forces along the perpendicular to the surface and they are called normal stresses and $(\sigma_{12}, \sigma_{13}, \sigma_{23})$ describes forces parallel to the surface and are called shear stresses which are discussed in more detail in the following subsection.

Generalized Hooke's law

The foundation of rheology in polymeric materials traces back to the classical laws of material behaviour. As mentioned earlier, in 1678, Robert Hooke introduced his famous law, encapsulated in the Latin phrase *ut tensio sic vis*, meaning "extension is proportional to the force" [18]. Hooke's law establishes the relationship between the applied force and the deformation of an elastic material. The concept of the stress tensor was introduced by Cauchy, who also gave the correct version of the constitutive equation for infinitesimal elasticity in 1827 which in general expressed as [18]:

$$\boldsymbol{\sigma} = \mathbf{C}\boldsymbol{\varepsilon}, \quad \sigma_{ij} = C_{ijkl}\varepsilon_{kl} \quad (2.5)$$

where $\boldsymbol{\sigma}$ is the stress tensor as described in 2.4, $\boldsymbol{\varepsilon}$ is the infinitesimal strain tensor as described in (2.3), and $\mathbf{C}$ is a 4th order elasticity tensor, a tensorial material constant, which reflects all the anisotropy of the material. For an isotropic solid, the elastic constants are reduced to just two:

$$C_{ijkl} = \lambda \delta_{ij} \delta_{kl} + \mu (\delta_{ik} \delta_{jl} + \delta_{il} \delta_{jk}) \quad (2.6)$$

where, λ and μ are called Lamé moduli [18]. Using (2.5) we can find:

$$\boldsymbol{\sigma} = \lambda(\text{tr } \boldsymbol{\varepsilon})\mathbf{I} + 2\mu\boldsymbol{\varepsilon}, \quad \sigma_{ij} = \lambda\varepsilon_{kk}\delta_{ij} + 2\mu\varepsilon_{ij} \quad (2.7)$$

if we take a traces of both side we have:

$$\text{tr } \boldsymbol{\sigma} = (3\lambda + 2\mu)\text{tr } \boldsymbol{\varepsilon} \quad (2.8)$$

by substituting this back to (2.7) we get:

$$\boldsymbol{\varepsilon} = \frac{1}{2\mu}\boldsymbol{\sigma} - \frac{\lambda}{2\mu(3\lambda + 2\mu)}(\text{tr } \boldsymbol{\sigma})\mathbf{I}, \quad \varepsilon_{ij} = \frac{1}{2\mu}\sigma_{ij} - \frac{\lambda}{2\mu(3\lambda + 2\mu)}\sigma_{kk}\delta_{ij} \quad (2.9)$$

In a uni-axial extension of a linear elastic material (2.7), the displacement field is given by

$$v_1 = \varepsilon x, \quad v_2 = -\nu \varepsilon y, \quad v_3 = -\nu \varepsilon z \quad (2.10)$$

where ε is the elongational strain, and ν is the amount of lateral contraction due to the axial elongation, called Poisson's ratio. If the lateral stresses are zero we would have:

$$\nu = \frac{\lambda}{2(\lambda + \mu)}, \quad \mu = \frac{E}{2(1 + \nu)} \quad (2.11)$$

where E is the Young's modulus. In other words, we have:

$$\sigma = E\varepsilon \quad (2.12)$$

Strains and stresses are often more effectively described using Young's modulus (E) or, in some cases, the bulk modulus, along with Poisson's ratio (μ). Young's modulus is a fundamental mechanical property that quantifies a material's stiffness, representing the ratio of stress to strain within the linear elastic range. It determines how much a material deforms under tensile or compressive forces, making it a crucial parameter in engineering and materials science. Poisson's ratio characterizes the tendency of a material to expand or contract in a direction perpendicular to an applied load. A higher Poisson's ratio indicates greater lateral deformation in response to axial stress. A value of 0.5 corresponds to a perfectly isotropic and incompressible material, meaning that any axial compression results in equal lateral expansion. This assumption is commonly used in biophysical measurements, particularly for biological tissues and soft materials where volume conservation is significant.

The uniaxial Hooke's Law relationship (2.12) is visualized in stress-strain curves, where materials under small strains exhibit a linear elastic region, characterized by proportionality between stress and strain. This behaviour ceases at the material's yield point, where permanent plastic deformation may occur [19] (Figure 2.2).

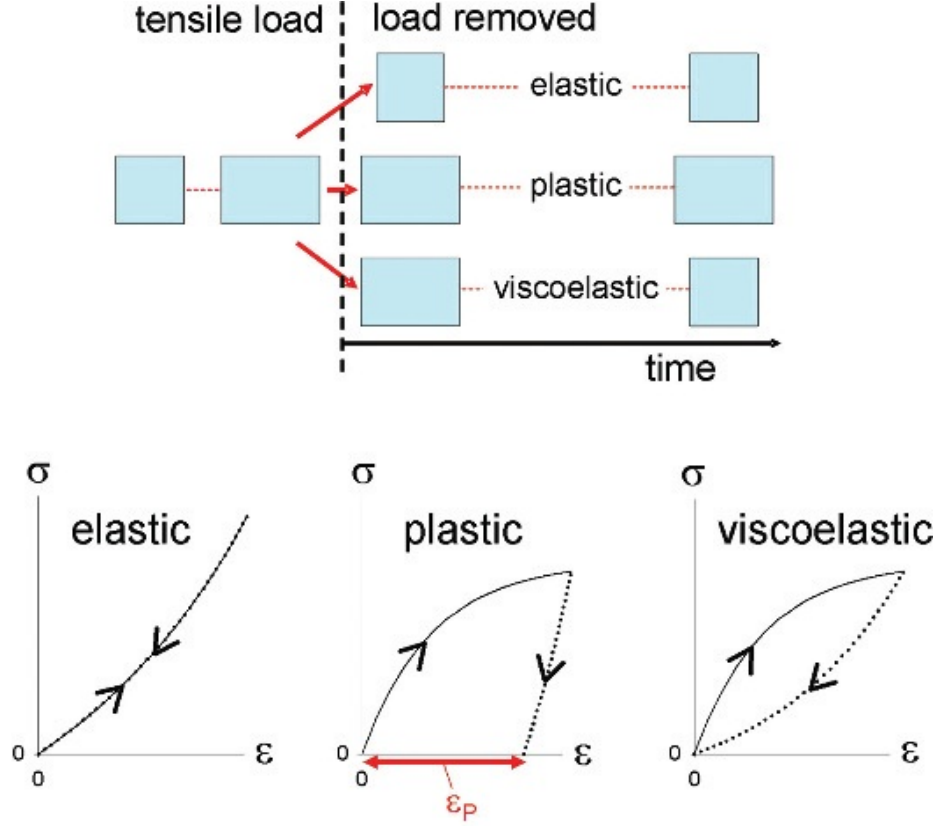


Figure 2.2: Stress-Strain Curves for different material. *Materials behave differently when the load is removed after deformation (left). An elastic material returns immediately to its original shape, a plastic material keeps the deformed shape forever and a viscoelastic material returns slowly to its original shape. Materials often have a combination of those properties: An elastoplastic material relaxes partially and retains only part of the deformation. Similarly, a viscoplastic material loses gradually a part of the deformation but a fraction of it stays forever. In a stress-strain experiment (right), the stress σ is measured as a function of strain ϵ . Full lines in the $\sigma - \epsilon$ curves show increasing stress and dotted lines decreasing stress. For elastic materials, these two lines coincide. If this line is non-linear (as occurs for many polymeric materials), the elasticity is called non-linear. Close to the origin (near zero stress), nearly all materials are linearly elastic. For plastic materials, a permanent strain ϵ_p remains after sufficiently large deformation. For viscoelastic materials, there is a hysteresis. The elastic modulus E is defined as the slope of the full line close to the origin [19]*

Shear Stress and Shear Strain

Building upon the previously discussed generalized Hooke's law, which relates stress and strain in elastic materials, we extend our focus to shear stress and shear strain, which are fundamental in describing multidirectional deformations. Unlike normal stresses ($\sigma_{11}, \sigma_{22}, \sigma_{33}$), which act perpendicular to a material's surface, shear stresses ($\sigma_{12}, \sigma_{13}, \sigma_{23}$) act tangentially, causing adjacent material layers to slide past each other. Shear behaviour is particularly relevant in biomechanical and polymeric materials, including hydrogels, where internal structural elements—such as polymer chains and crosslinks—respond differently under shear compared to uniaxial tension.

Shear stress (τ) is the force per unit area applied tangentially to a material's surface, causing layers to slide relative to each other. It is defined as:

$$\tau = \frac{F}{A} \quad (2.13)$$

where τ is the shear stress (Pa), F is the applied force parallel to the surface (N), and A is the cross-sectional area over which the force acts (m^2). Shear strain (γ) quantifies the angular distortion

caused by shear stress and is defined as:

$$\gamma = \tan \theta \approx \theta \quad (2.14)$$

for small angles, where θ is the angular displacement in radians.

For linear elastic materials, shear stress and shear strain are related by Hooke's law for shear:

$$\tau = G\gamma \quad (2.15)$$

where G is the shear modulus, which characterizes a material's resistance to shear deformation.

For an isotropic material, the shear modulus is related to Young's modulus (E) and Poisson's ratio (ν) through:

$$G = \frac{E}{2(1 + \nu)} \quad (2.16)$$

This equation highlights that a material's shear behaviour is intrinsically linked to its overall elastic properties.

For materials exhibiting viscoelastic behaviour, such as polymers and biological tissues, the response to applied stress is both elastic and viscous, leading to time-dependent deformation. To characterize this behaviour, especially under oscillatory loading conditions, the complex shear modulus (G^*) is introduced:

$$G^*(\omega) = G'(\omega) + iG''(\omega) \quad (2.17)$$

here, ω represents the angular frequency of the applied oscillatory stress, and i is the imaginary unit.

G' represents the elastic portion of the material's response, indicating the energy stored and recovered per cycle of deformation. It measures the material's stiffness and is given by:

$$G' = \frac{\sigma_0}{\gamma_0} \cos \delta \quad (2.18)$$

where σ_0 and γ_0 are the amplitudes of stress and strain, respectively, and δ is the phase angle between them.

G'' represents the viscous portion, reflecting the energy dissipated as heat during deformation. It is calculated as:

$$G'' = \frac{\sigma_0}{\gamma_0} \sin \delta \quad (2.19)$$

in this context, G'' quantifies the material's internal friction and viscous flow characteristics.

Loss factor

In purely elastic materials, all energy stored during deformation is fully recovered upon unloading. However, in viscoelastic materials such as hydrogels, biological tissues, and polymers, some of this energy is dissipated as heat due to internal molecular friction. This energy dissipation is captured by the loss factor ($\tan \delta$), which quantifies the ratio of energy lost to energy stored per deformation cycle. The loss factor is defined as:

$$\tan \delta = \frac{G''}{G'} \quad (2.20)$$

where G' is the storage modulus (elastic response), representing the material's ability to store mechanical energy, and G'' is the loss modulus (viscous response), indicating the energy dissipated as heat.

A higher loss factor ($\tan \delta$) means that a material exhibits greater energy dissipation, behaving more like a viscous fluid, while a lower loss factor indicates a more elastic response. This parameter is crucial in biomechanics, damping materials, and polymer science, where energy absorption and dissipation play a vital role in mechanical stability.

Understanding shear behaviour and the loss factor is essential for predicting the mechanical response of soft materials such as hydrogels and biological tissues, which exhibit both solid-like (elastic) and fluid-like (viscous) properties. The loss factor is particularly significant in dynamic mechanical analysis (DMA), where materials are subjected to oscillatory shear stress to evaluate their energy storage and dissipation capabilities.

By incorporating shear stress, shear strain, and the loss factor within the generalized Hooke's law framework, mechanical behaviour of complex materials can be modeled effectively.

Viscosity

Viscosity, is defined as the ratio of shear stress to shear rate in simple shear flow:

$$\eta = \frac{\tau}{\dot{\gamma}}. \quad (2.21)$$

where τ is the shear stress and $\dot{\gamma}$ is the shear rate.

Given that hydrogels (> 90%) and soft tissues (> 70%) are predominantly composed of water, viscosity plays a crucial role in their mechanical behaviour[20, 21]. In hydrogels, it influences viscoelastic properties, affecting their ability to deform and recover under stress. Similarly, in soft tissues, viscosity governs fluid movement, impacting nutrient transport and cellular interactions. The viscosity is a complex function under oscillatory conditions and can be defined as:

$$\eta^*(\omega) = \eta'(\omega) - i\eta''(\omega) \quad (2.22)$$

$$\eta^*(\omega) = \frac{G^*(\omega)}{i\omega} \quad (2.23)$$

$$\eta' = \frac{G''}{\omega} \quad (2.24)$$

$$\eta'' = \frac{G'}{\omega} \quad (2.25)$$

where the η' is commonly referred to as the dynamic viscosity and η'' represents the elastic viscosity.

Material Classification in Rheology: Elastic, Viscoelastic, and Poroelastic Responses

While Hooke's law effectively describes elastic solids, many real-world materials, including polymers, exhibit a combination of elasticity and viscosity, leading to viscoelasticity. Unlike purely elastic materials where deformation is instantaneous and reversible, viscoelastic materials display time-dependent deformation. In such cases, the stress is influenced not only by the strain but also by the strain rate [19] as explained in 2.21.

This dual behaviour is a hallmark of polymers and is central to rheology. Unlike purely elastic materials, viscoelastic materials dissipate part of the stored mechanical energy as heat [22]. This energy loss is observed in materials such as polymers and biological tissues, which exhibit different stress-strain behaviours depending on the rate at which strain is applied. Soft tissues, for instance, display strain stiffening, meaning that their stiffness increases as strain rate increases. This behaviour makes viscoelastic materials highly sensitive to experimental conditions, requiring precise control over strain rates to ensure reproducible measurements.

Another type of material is poroelastic [23]. Poroelastic materials consist of a solid elastic matrix interspersed with a fluid-filled porous network. Their mechanical response is influenced by both the deformation of the solid framework and the movement of fluid within the pores [24]. When stress is applied, the solid structure deforms, creating pressure differences that drive fluid migration. This interaction between solid deformation and fluid movement results in time-dependent behaviour similar to viscoelasticity. However, unlike viscoelastic materials, where time dependence arises from molecular rearrangements, poroelastic behaviour is governed by the rate of fluid redistribution [24].

Poroelectric effects are particularly significant in hydrated biological tissues such as cartilage, as well as in engineered hydrogels and porous geological materials. The relaxation of stress in poroelectric materials depends on their characteristic length scale, meaning that larger samples exhibit different relaxation trends compared to smaller ones. This property can be exploited to distinguish poroelectric effects from viscoelasticity using multi-scale indentation experiments, which analyze how relaxation changes with sample size. The key distinction between viscoelasticity and poroelectricity lies in their respective mechanisms of energy dissipation and time-dependent deformation. Viscoelastic materials dissipate energy through internal molecular friction, whereas poroelectric materials dissipate energy due to fluid flow within the porous network. Moreover, viscoelasticity is independent of sample size, while poroelectricity is strongly influenced by the characteristic length of the material [22].

Understanding the differences between elastic, viscoelastic, and poroelectric materials is essential for various applications in engineering, biology, and material science. While elastic materials provide simple and instantaneous mechanical responses, viscoelastic and poroelectric materials introduce complex, time-dependent behaviours that must be considered when designing structures, medical implants, or biomaterials. Choosing the correct material model whether elastic, viscoelastic, or poroelectric is critical for accurately predicting mechanical behaviour in real-world applications such as tissue engineering, hydrogel development, and structural mechanics.

Generally, polymer scaffolds behave in a more elastic fashion in response to a rapidly applied force and in a more viscous fashion in response to a slowly applied force [25]. As explained earlier viscoelasticity means behaviour similar to both purely elastic solids in which the deformation is proportional to the applied force and to viscous material in which the rate of deformation is proportional to the applied force. Therefore, their response to stress or strain can be categorized into two regimes: linear viscoelasticity and nonlinear viscoelasticity, depending on the magnitude of the applied deformation and the material’s intrinsic properties. As a result, The behaviour of polymers is very complex due to this dual nature.

In the linear viscoelastic regime, the material’s response to stress or strain is directly proportional to the applied load. This implies that the relationship between stress and strain is linear, and the material’s properties remain constant regardless of the magnitude of the applied force, provided it is within a small deformation range. Mathematically, this behaviour can be described using linear differential equations, and the principle of superposition applies, meaning the total response caused by multiple stimuli is the sum of the responses that would have been caused by each stimulus individually. Linear viscoelasticity is typically observed under small deformations where the material structure is not significantly altered.

When the applied stress or strain exceeds a certain threshold, the material enters the nonlinear viscoelastic regime. In this state, the relationship between stress and strain becomes nonlinear, and the material properties may change with the level of applied deformation. Nonlinear viscoelastic behaviour is characterized by phenomena such as strain stiffening or softening, where the material becomes respectively harder or softer to deform as the strain increases [26].

2.3.3 Maxwell and Kelvin-Voigt models

One of the approaches for mechanical modeling of viscoelastic material and force profiles generated using micromanipulation is to construct models consisting of spring and dashpot elements [25, 27]. The spring complies with Hooke’s law as a mechanical model with an elastic element. When a spring is fixed at one end and a load is applied at the other, it becomes instantaneously extended. Then, after the load is removed, it immediately recovers its original length. On the other hand, the dashpot complies with Newton’s law of viscosity as a mechanical model with a viscous element. When a load is applied to a dashpot, it opens gradually, strain being a function of time. Then, after the load is removed, the dashpot remains open and recovery does not occur. The mechanical model which consists of a Hookean spring and a Newtonian dashpot in parallel is a Kelvin-Voigt element (Figure 2.3a), and that in series is a Maxwell element (Figure 2.3b). These simple models form the basis for explaining the behaviour of polymers, which can be described by combining them [19].

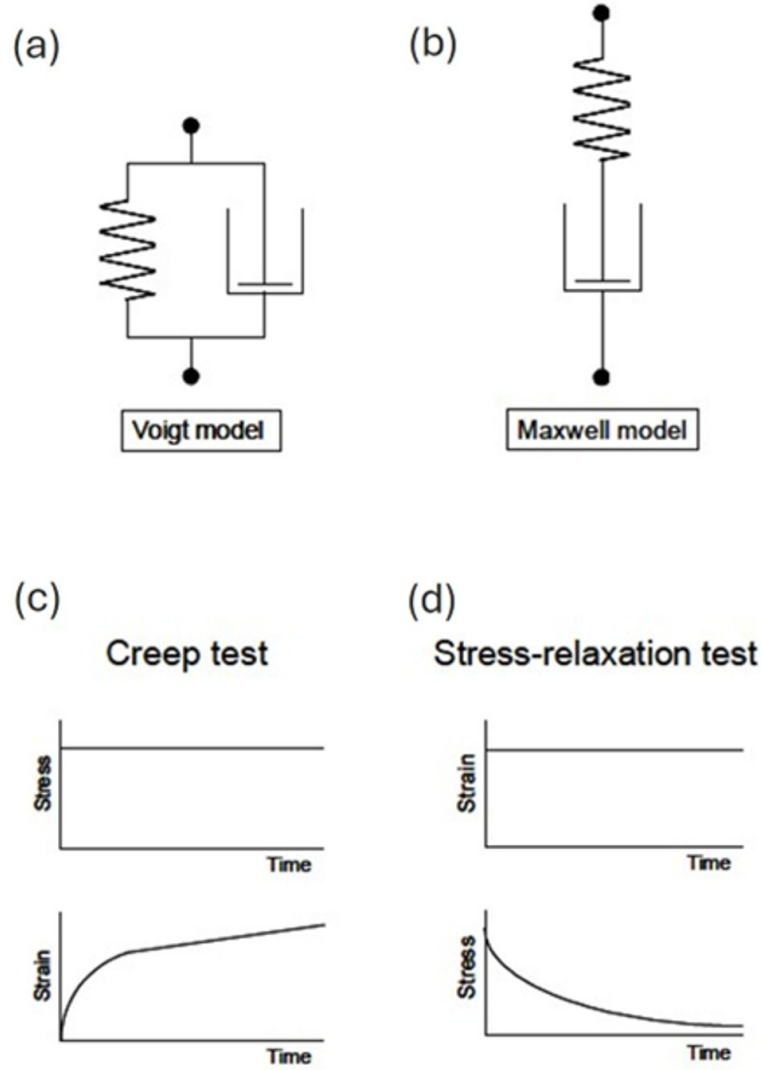


Figure 2.3: Viscoelastic tests and models. a) *Maxwell model*; b) *Voigt model*; c) *Stress relaxation test*; d) *Creep test* [25]

The concept of linear viscoelasticity was originated with Maxwell , who proposed the following equation in 1867–68:

$$\frac{d\sigma}{dt} = E \frac{d\varepsilon}{dt} - \frac{\sigma}{\lambda} \quad (2.26)$$

where λ (relaxation time) is a time constant. When the relaxation time is zero, keeping the product constant, the Newtonian model is recovered. And when the relaxation is infinitely large, a further integration yields the Hookean model

consequently, in the context of shear deformation we have:

$$\frac{d\tau}{dt} + \frac{\lambda}{\tau} = G \frac{d\gamma}{dt} \quad (2.27)$$

In the Kelvin-Voigt model, a dashpot is assumed to act in parallel with an elastic spring. As a

result, the deformation equation incorporates both the strain ε and its time derivative ($\frac{d\varepsilon}{dt}$), and is expressed as:

$$\sigma = E\varepsilon + \eta \frac{d\varepsilon}{dt} \quad (2.28)$$

consequently, in the context of shear deformation we have:

$$\tau = G\gamma + \eta \frac{d\gamma}{dt} \quad (2.29)$$

Combining the solid response (G, γ) and liquid response (τ) in one equation.

2.3.4 Creep and stress relaxation tests

Creep and stress relaxation tests are fundamental methods used to characterize the time-dependent mechanical behaviour of materials, particularly those exhibiting viscoelastic properties. Creep occurs when a material continues to deform over time under a constant applied stress (Figure 2.3c), while stress relaxation refers to the gradual reduction of internal stress when held at a constant deformation (Figure 2.3d). Creep test is essential for understanding how materials deform under long-term loading conditions.

Creep test

Creep deformation in materials under constant stress occurs in three distinct stages. In primary creep, the strain increases rapidly at first, but the rate of deformation gradually decreases due to work hardening. Secondary creep follows, where the strain rate becomes nearly constant as a balance between work hardening and recovery processes is established. Finally, tertiary creep occurs, in which the strain rate accelerates significantly, leading to material failure due to mechanisms such as necking, void formation, or microstructural breakdown. Understanding these stages is essential for predicting how materials behave under long-term loading conditions.

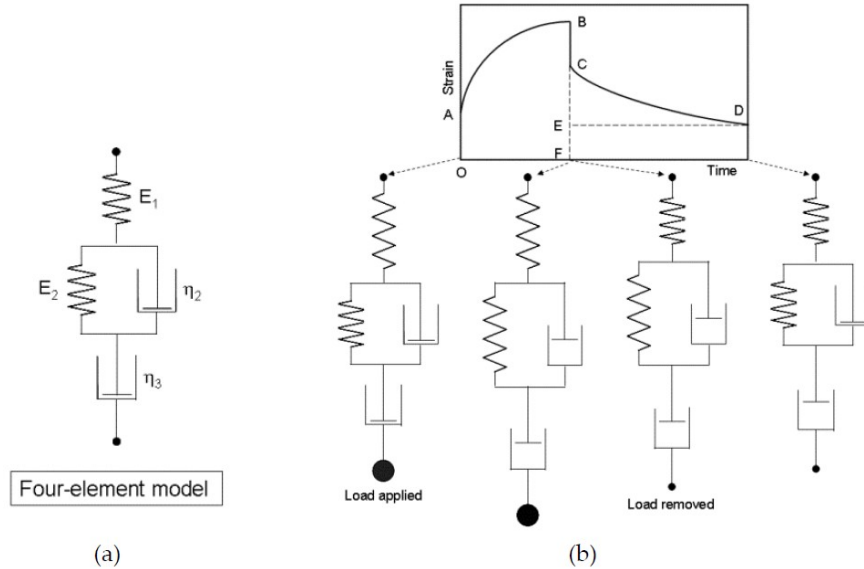


Figure 2.4: Creep and recover creep test analysis. (a) Four-element model for creep and creep recovery; (b) creep and creep recovery. OA: Instantaneous strain; AB: Time dependent opening; BC: Instantaneous recovery ($=OA$); CD: Slow recovery; EF: Permanent deformation[25]

The time-dependent behaviour of creep and creep recovery can be effectively analyzed using a four-element mechanical model (Figure 2.4), which consists of two springs (E_1, E_2) and two dashpots

(η_2, η_3) . This model helps explain how materials deform under sustained stress and how they recover once the stress is removed. When a constant stress is applied at $t = 0$, the material exhibits an instantaneous elastic strain due to the spring E_1 , followed by a gradual increase in strain caused by the combined behaviour of the spring E_2 and dashpots (η_2, η_3) . This gradual increase in strain corresponds to the primary and secondary creep phases, where dashpots introduce time-dependent deformation. As long as the stress is maintained, the strain continues to develop at a steady rate, representing secondary creep.

When the applied stress is removed, the material undergoes creep recovery, which consists of two main components. The instantaneous recovery corresponds to the response of the elastic spring E_1 , which allows the material to regain part of its original shape immediately. Then, a gradual recovery occurs due to the spring-dashpot pair (E_2/η_2) , representing delayed elastic effects. However, due to the fully viscous nature of dashpot η_3 , some deformation remains permanent, meaning that the material does not return completely to its original shape. This irreversible strain corresponds to the effects observed in tertiary creep, where structural damage and viscous flow lead to permanent deformation.

By integrating the three stages of creep with the four-element model, it becomes clear how different components contribute to elastic deformation, time-dependent viscous flow, and permanent strain accumulation. This understanding is essential for predicting the long-term mechanical behaviour of materials in engineering applications, polymer science, and biomechanics.

Stress relaxation test

A stress relaxation test is a method used to analyze the time-dependent behaviour of materials under constant strain. When considering the response of a material to deformations, both the characteristic timescales of the material and the timescale of the deformation are of key importance. The characteristic timescale of a material is the time it takes the material to adjust to an equilibrium stress state after applying a deformation. These timescales characterize the relaxation of stress in the material and are therefore referred to as relaxation times. For many soft materials, relaxation times typically range from milliseconds to minutes, aligning with the timescales of most practical deformations. This overlap contributes to the rich and complex rheological behaviour observed in soft matter [28].

In this test, a material is rapidly deformed to a specific strain level and then held at that deformation while the decay of stress over time is recorded. The gradual decrease in stress occurs as the material undergoes internal structural adjustments, allowing for the redistribution of molecular forces. This phenomenon is particularly relevant in viscoelastic materials, where both elastic and viscous components influence the stress response.

Stress relaxation tests are crucial for characterizing polymer behaviour, especially in applications where materials are expected to maintain their mechanical integrity under sustained deformation. Polymers, for example, exhibit different stress relaxation responses depending on whether they are cross-linked or uncross-linked [25]. The stress relaxation behaviour of cross-linked amorphous polymers differs significantly from that of uncross-linked amorphous polymers due to differences in molecular mobility.

In cross-linked amorphous polymers, molecular chains are chemically bonded, preventing flow and restricting large-scale molecular rearrangements. As a result, their stress relaxation behaviour is best described using a three-element model, consisting of one Maxwell element and an additional parallel spring (Figure 2.5). This model effectively represents the limited molecular motion and long-term stability of cross-linked materials.

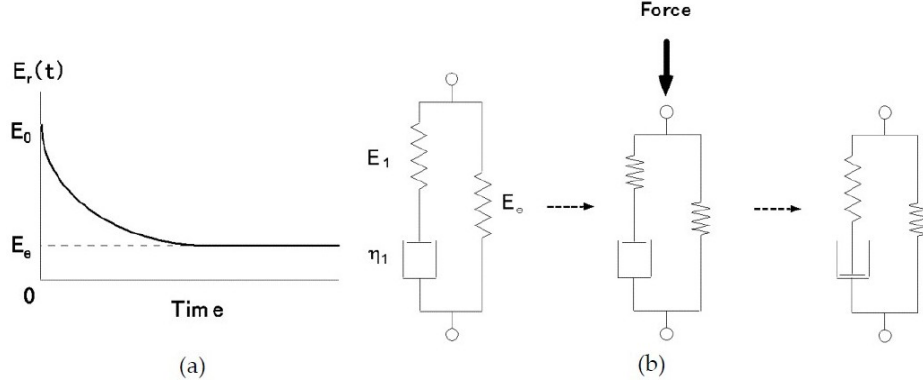


Figure 2.5: Stress relaxation test analysis. (a) Stress relaxation curve of cross-linked polymers; (b) Three-element model in which one Maxwell element and one spring are connected in parallel [25]

Conversely, uncross-linked amorphous polymers lack permanent chemical bonds between chains, allowing for significant molecular flow over time. This ability to flow leads to more pronounced stress relaxation effects. To describe this behaviour, a four-element model is used, consisting of two Maxwell elements connected in parallel (Figure 2.6). This model accounts for both short-term elastic response and long-term viscous flow, providing a more accurate representation of how stress dissipates in uncross-linked polymers.

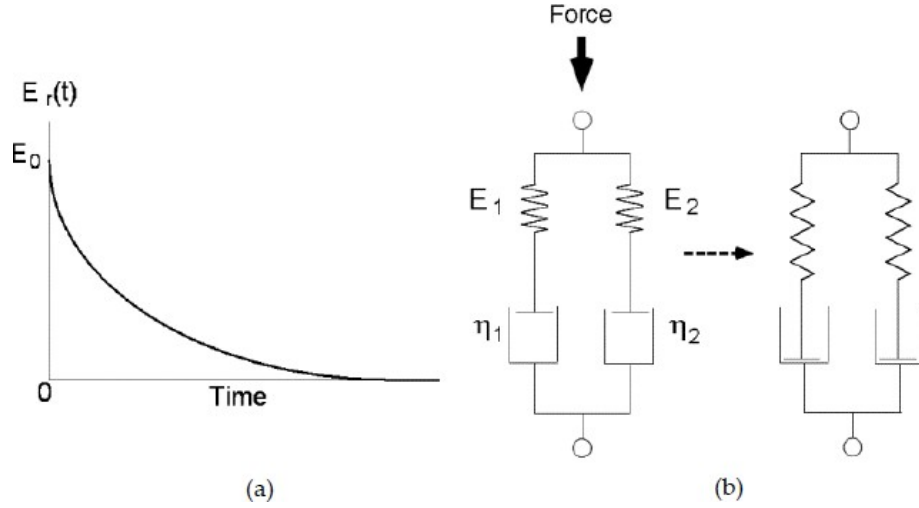


Figure 2.6: Stress relaxation test analysis. (a) Stress relaxation curve of uncross-linked polymers; (b) Four-element model in which two Maxwell elements are connected in parallel[25]

2.3.5 The Boltzman superposition principle (General equation of linear viscoelasticity)

The Boltzmann Superposition Principle is a fundamental concept in linear viscoelasticity, addressing the limitations of classical models like Maxwell and Kelvin-Voigt, which fail to fully describe the complex time-dependent behaviour of real materials. While the Maxwell model predicts stress relaxation but not creep, and the Kelvin-Voigt model captures creep but not stress relaxation, Boltzmann proposed a more general framework to account for the history-dependent nature of viscoelastic responses [18, 29].

Boltzmann introduced the idea that the current stress in a viscoelastic material is influenced not only by the present strain but also by all past strains. However, older strains contribute less to the

current stress than more recent deformations, a concept known as fading memory. Mathematically, this idea is represented as an integral that accounts for the cumulative effect of all past deformations.

If an infinitesimal strain increment $d\gamma(t')$ occurs at time t' , it contributes to the total stress at a later time t according to a stress relaxation function $G(t - t')$, which describes how stress dissipates over time. The total stress at time t is then given by the integral:

$$\tau(t) = \int_{-\infty}^t G(t - t') \frac{d\gamma(t')}{dt'} dt' \quad (2.30)$$

where each past strain increment contributes independently to the total stress. This linearity ensures that the response to multiple deformations is simply the sum of responses to each individual deformation, reinforcing the memory effect in viscoelastic materials.

The Newtonian liquid is recovered with the delta memory function [18]

$$G(t - t') = \eta_0 \delta(t - t') \quad (2.31)$$

$$\tau(t) = \eta_0 \frac{d\gamma(t)}{dt} \quad (2.32)$$

The Boltzmann Superposition Principle provides a general equation for linear viscoelasticity, incorporating both creep and stress relaxation. By integrating the effects of strain history through memory functions, this principle allows for accurate modeling of viscoelastic materials under real-world conditions, where deformations occur over extended time scales and involve multiple loading sequences.

2.3.6 Rheometer and Sweep tests

A rheometer (Figure 2.7a) is a highly developed instrument used to measure the rheological properties of materials, particularly their response to applied forces in terms of deformation and flow. It offers a comprehensive analysis of both viscous and elastic behaviours across different stress, strain, and temperature conditions. This capability makes them essential in studying complex fluids and soft solids, including polymers, biological tissues, and hydrogels [30].

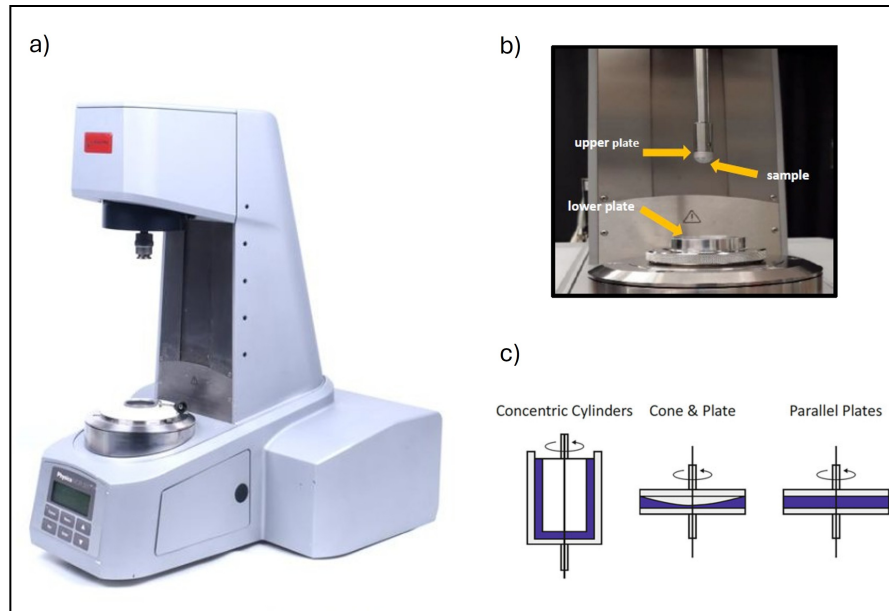


Figure 2.7: Rheometer. (a) MCR 301 Anton Paar rheometer; (b) Experimental setup with a parallel plate; (c) Schematic of the geometrical plates [31].

The core structure of a rheometer consists of a drive motor and encoder, which precisely control and measure torque, angular displacement, and rotational speed. A force transducer is responsible for measuring the stress exerted on the sample, while the test chamber houses different measurement geometries, depending on the nature of the material being tested. The choice of geometry significantly influences the accuracy of measurements and the type of data collected (Figure 2.7c).

Several measurement configurations are commonly used in rheometers. The cone-and-plate geometry features a shallow cone positioned just above a flat plate, with the sample confined between them. This setup ensures a uniform shear rate across the sample and is ideal for measuring viscosity and viscoelastic properties of low to medium viscosity fluids. The parallel plate geometry, in contrast, consists of two flat plates with the sample in between, making it well-suited for soft solids, hydrogels, and pastes (Figure 2.7b). The coaxial cylinder (Couette) geometry uses two concentric cylinders, with the fluid occupying the annular space between them. In this configuration, one cylinder rotates while the other remains stationary, generating a controlled shear flow, making it useful for measuring suspensions and highly viscous liquids [30].

Rheometers operate primarily in two modes: rotational (steady shear) mode and oscillatory (dynamic) mode. In rotational mode, the instrument applies a constant shear rate or shear stress to the sample and measures the corresponding response. This mode is particularly useful for determining flow curves and understanding behaviours such as shear-thinning, shear-thickening, or yield stress properties. In oscillatory mode, the rheometer subjects the sample to sinusoidal deformations and measures its response over time, allowing for the characterization of viscoelastic properties. This mode provides insight into the material's storage modulus and loss modulus, which represent the elastic and viscous components, respectively [30].

In our laboratory, we used the MCR 301 Anton Paar rheometer to analyze the rheological properties of our samples. This strain-controlled rheometer (SSC) was employed using plate-plate geometry, where the samples were positioned between two parallel stainless steel sandblasted plates, separated by a precisely controlled gap and immersed in phosphate-buffered saline (PBS) to maintain hydration.

Oscillation Amplitude Sweep

The amplitude sweep experiment (Figure 2.8) is a widely used rheological test to investigate the Linear Viscoelastic (LVE) region and identify the transition into nonlinear behaviour [32]. The two-plate model is used to describe the oscillatory motion applied to the sample. In this measurement, the sample is placed between two parallel plates, where the upper plate oscillates sinusoidally while the lower plate remains stationary. The upper plate moves back and forth at a constant angular frequency while the strain amplitude systematically increases throughout the experiment. Since the upper plate moves in an oscillatory motion, the applied shear strain follows a sinusoidal function:

$$\gamma(t) = \gamma_0 \sin(\omega t) \quad (2.33)$$

where γ_0 is the strain amplitude, ω is the angular frequency, and t is time.

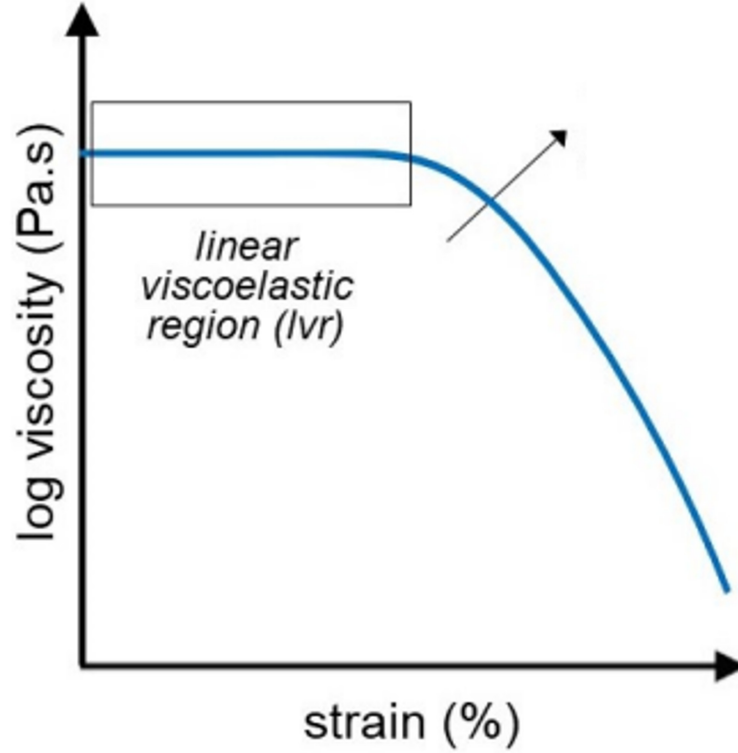


Figure 2.8: Amplitude sweep experiment. *Strain sweep or amplitude sweep in hydrogels shows the linear viscoelastic region (LVR) [33].*

In amplitude sweep experiments, the strain amplitude gradually increases while keeping the oscillation frequency constant. The primary objective is to determine the LVE limit, which marks the transition from linear viscoelastic behaviour to nonlinear response, and to locate the yield point, where the material begins to exhibit irreversible structural changes.

In the two-plate model, both plates are equipped with sensors to detect and record material response. The first sensor measures the applied strain on the upper plate, referred to as the angle of torque. This signal is plotted as a sinusoidal function over time, with increasing strain amplitude as the experiment progresses. The oscillation period remains constant, with the oscillation frequency defined as the reciprocal of the period.

The second sensor detects the torque acting on the lower plate, which counteracts the applied force to keep the plate in position. This torque is recorded as the shear stress response of the material. The stress response is also sinusoidal but with a phase shift relative to the applied strain:

$$\tau(t) = \tau_0 \sin(\omega t + \delta) \quad (2.34)$$

where τ_0 is the stress amplitude, and δ represents the phase angle between stress and strain.

The phase difference δ provides insight into the material's behaviour. For purely elastic materials, stress and strain are in phase. Therefore ($\delta = 0^\circ$), For purely viscous materials, stress lags strain by 90° ($\delta = 90^\circ$), and for viscoelastic materials, the phase angle lies between 0° and 90° , reflecting a combination of elastic and viscous contributions.

In the LVE region, both the storage modulus and loss modulus remain constant, indicating that the material structure remains unchanged despite increasing strain amplitude. The LVE occurs at low shear stress, where the moduli are independent of the increasing stress. However, as the strain surpasses a critical threshold, G' begins to decrease, marking the end of the LVE region and the onset of structural breakdown. This point indicates the yielding behaviour of the material. At

even higher strain values, a crossover between G' and G'' may be observed, which corresponds to the gel-sol transition, indicating that the material has transitioned from a solid-like to a liquid-like state.

The amplitude sweep test is particularly useful for characterizing hydrogels and determining their stability limits. Studies have shown that hydrogel composition influences the extent of the LVE region and the presence of a crossover point. For example, cellulose nanofiber hydrogels with different weight percentages (wt.%) of fibers exhibited varying LVE ranges, with higher fiber content leading to a shorter LVE region and a more distinct crossover point. In some cases, hydrogels with very low fiber content (e.g., 0.09 wt.%) did not show a crossover point within the tested stress range, indicating stronger gel-like behaviour [33].

Additionally, amplitude sweeps have been used to study the self-healing properties of certain hydrogels. Some materials, such as PHEMA-based hydrogels, exhibit the ability to recover their original mechanical properties after being subjected to high strain [33]. Once the strain is reduced back to the LVE stress level, these hydrogels regain nearly 97% of their initial G' and G'' , demonstrating their self-healing behaviour.

The amplitude sweep experiment provides essential information about a material's structural stability, resistance to deformation, and ability to recover after stress application. Identifying the LVE region and yield point helps in optimizing material formulations for industrial applications in food science, pharmaceuticals, cosmetics, and biomaterials. By systematically increasing strain amplitude while maintaining a constant frequency, amplitude sweeps allow researchers to understand the limits of viscoelastic behaviour and predict mechanical performance under real-world conditions.

Oscillation Frequency Sweep

The frequency sweep experiment is a fundamental rheological method used to investigate the relationship between angular frequency and the viscoelastic properties of a material. This test measures the storage modulus and loss modulus across a range of frequencies while keeping the strain amplitude constant within the LVR to ensure non-destructive deformation. Higher frequencies correspond to shorter timescales, capturing the material's immediate response to deformation, whereas lower frequencies represent longer timescales, revealing its long-term structural stability. The primary objective of the frequency sweep experiment is to analyze the frequency dependence of viscoelastic materials and determine their elastic or viscous nature. The test provides key insights into the inner structure of materials, including polymers, hydrogels, and biomaterials, helping researchers distinguish between different types of crosslinked and uncrosslinked systems [32].

In an oscillatory test, by considering 2.33 and 2.34, the G' and G'' can be defined as:

$$G' = \frac{\tau_0}{\gamma_0} \cos(\delta), \quad G'' = \frac{\tau_0}{\gamma_0} \sin(\delta) \quad (2.35)$$

The frequency sweep experiment provides three characteristic viscoelastic responses based on the behaviour of G' and G'' (Figure 2.9). For viscoelastic solids, G' remains constant at low frequencies and dominates over G'' . At high frequencies, G'' increases but never surpasses G' , indicating solid-like behaviour at all timescales. Examples include toothpaste and lotions, which maintain their structure but flow under sufficient force. In viscoelastic liquids G'' is greater than G' at low frequencies ($G'' > G'$), indicating that the material behaves like a liquid over long timescales. However, at higher frequencies, the storage modulus dominates ($G' > G''$), suggesting that the material temporarily stores energy like a solid. The $G' - G''$ crossover point (ω_{cross}) marks the relaxation time, where stored elastic stresses dissipate into viscous flow. Common examples of viscoelastic liquids include shampoos, glues. Finally, for gel-like materials, G' and G'' remain parallel across the frequency range without a crossover, indicating a stable network structure. Gels exhibit a well-defined internal polymer or colloidal network that maintains its solid-like integrity. Examples include agarose hydrogels and biological tissues.

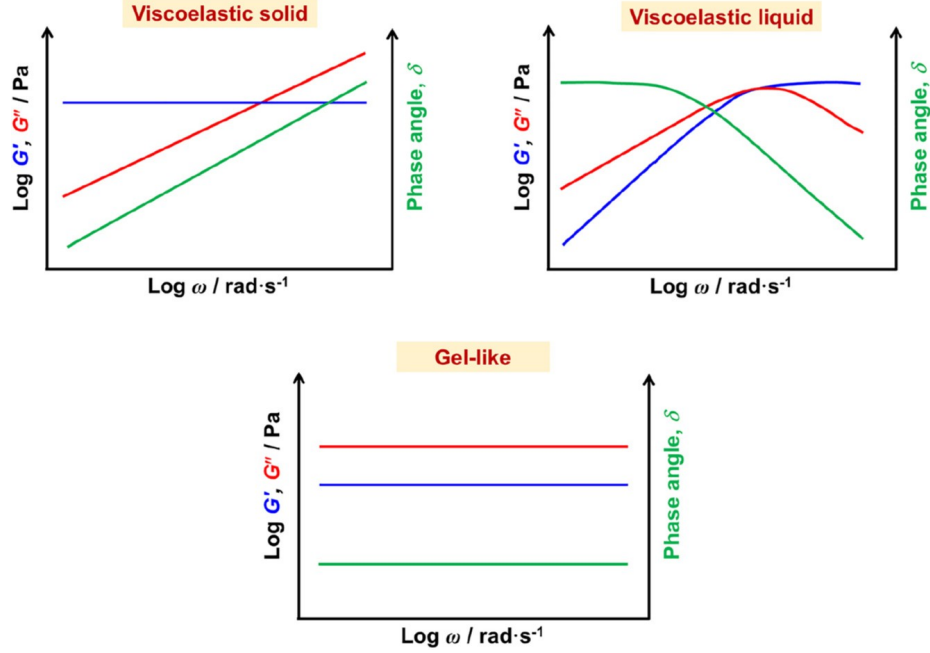


Figure 2.9: Frequency sweep experiment. Typical frequency response in an oscillatory at constant amplitude for viscoelastic solid, viscoelastic liquid and gel materials[32].

By analyzing how storage and loss moduli vary across different frequencies, researchers can distinguish between solid-like, liquid-like, and gel-like responses. The test is particularly valuable for understanding crosslinking mechanisms, additive effects, and structural transitions, providing critical insights for material design in biomedicine, food science, and industrial applications [32].

In this thesis, amplitude sweep and frequency sweep experiments were performed to study the mechanical behaviour of collagen-based hydrogels and to investigate how cell presence and other parameters influence their mechanical properties. These experiments were conducted at different frequencies and strain amplitudes to analyze the viscoelastic responses of the hydrogels under varying conditions. The results obtained from these tests provide essential insights into how different factors, including cell interactions, affect the structural and mechanical stability of the hydrogels. The detailed findings and their implications will be presented in the following chapters.

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Chapter 3

Scaffold Fabrication and Experimental Design

3.1 Background

Tissue engineering is an interdisciplinary field focused on designing biological substitutes that restore, maintain, or model tissue function [1, 2]. While traditionally associated with regenerative medicine, tissue engineering has also emerged as a valuable research platform for studying complex tissue behaviours in vitro. A central challenge in tissue engineering is the ability to fabricate environments that mimic the ECM.

Developing such in vitro environments requires precise control over scaffold composition, structure, and mechanical properties. Fabrication methods are therefore critical, as they determine not only the physical characteristics of the scaffold but also how cells respond to those cues. For example, fibroblasts, key ECM-regulating cells in both wound healing and pathological conditions, alter their morphology and function based on matrix stiffness, fiber alignment, and biochemical composition.

In disease research, tissue engineered models have been used to replicate tumor microenvironments, offering insights into cancer progression and therapeutic response [3]. However, the utility of tissue engineering platforms extends far beyond oncology. They allow researchers to isolate and study specific cellular behaviours in tunable conditions that are difficult to reproduce in vivo. In this work, temperature-controlled casting methods were used to fabricate 3D collagen–fibronectin hydrogels, which serve as simplified ECM models to study how fibroblast cells interact with, and remodel, their surrounding matrix.

In vitro models that more accurately replicate in vivo tissue environments are essential for studying cell behaviour under physiologically relevant conditions. While traditional two-dimensional (2D) culture systems offer simplicity and ease of use, they fail to capture the complexity of native ECM interactions. Three-dimensional (3D) culture platforms, in contrast, provide a more realistic representation of tissue architecture, mechanical properties, and cellular signaling environments [4, 3]. For this reason, 3D scaffolds have become central to tissue engineering studies where understanding cell–matrix interactions and tissue-level behaviours is essential. In this work, 3D collagen-based hydrogels were used to create defined ECM-like environments in which fibroblast behaviour could be studied under controlled conditions.

To replicate the complexity of the ECM in three dimensions, a variety of in vitro models have been developed, each offering distinct advantages and limitations depending on the intended application. These include spheroids, organoids, organ-on-a-chip platforms, decellularized ECM matrices, bio-printed constructs, and scaffolds ranging from porous solids to soft hydrogel-based systems [4, 3, 5].

Spheroids and organoids are scaffold-free or partially scaffold-assisted 3D culture systems that have gained widespread use in biomedical research, particularly in cancer biology. Spheroids are self-assembled aggregates of cells that promote strong cell-cell interactions and develop physiologically relevant gradients of oxygen, nutrients, and waste, thus partially mimicking in vivo conditions [4,

6, 7]. They are cost-effective and suitable for high-throughput assays, making them valuable for early-stage drug screening and tumor modeling [4]. However, their lack of a structured ECM limits their ability to fully replicate the complex tumor–matrix interface [4].

Organoids build upon the spheroid model by incorporating organ-specific stem or progenitor cells that self-organize into more complex structures, often resembling the architecture and function of the tissue of origin [4, 8]. They can be cultured with or without scaffold support and offer improved cellular heterogeneity and relevance for personalized medicine [9, 4]. Despite these advantages, organoids still face challenges including high variability, limited reproducibility, and incomplete modeling of tumor–stroma interactions [4].

Another method for modeling tissue microenvironments in vitro is the organ-on-a-chip (OoC) platform, which integrates microfluidics, biomaterials, and cell culture to create dynamic models that closely mimic human tissue architecture and physiological responses [10, 11]. ...

Organ-on-a-chip (OoC) technology integrates microfluidics, biomaterials, and cell culture to create dynamic in vitro models that closely mimic human tissue architecture and physiological responses [10, 11]. By incorporating living cells into microfabricated channels, OoC devices enable precise control over biochemical and mechanical stimuli, such as shear stress, nutrient gradients, and flow dynamics—factors critical for replicating organ-level function [10, 11, 3].

Unlike static 2D cultures or self-assembling organoids, OoCs allow for real-time monitoring and mechanical stimulation within a controlled microenvironment. These systems are built using biocompatible materials, often structured through either top-down or bottom-up tissue formation strategies, and can support co-cultures to model complex interactions such as the immune response or vascular transport [12, 11].

Despite their potential, OoC systems face limitations such as fabrication complexity, low throughput, and material constraints, especially with widely used polymers like PDMS, which can absorb small molecules and limit scalability [13, 11]. Advances in automation, materials, and integrated sensing technologies are helping to address these issues, paving the way for broader adoption in biomedical research and pharmaceutical development [13].

These alternative systems are reviewed here to contextualize the rationale for selecting scaffold-based hydrogels as the experimental focus of this thesis. Among these 3D modeling strategies, scaffold-based systems, particularly hydrogels composed of ECM proteins such as collagen and fibronectin, offer a unique balance of biological relevance, tunability, and experimental accessibility.

3.2 Scaffolds

Scaffolds are essential components in tissue engineering, serving as 3D structures designed to replicate the ECM environment and support critical cellular activities such as adhesion, proliferation, differentiation, and ECM production. Scaffolds are fundamental not only in common tissue engineering but also in advanced biological models. These constructs are intended to mimic the architecture, biological cues, and mechanical properties of native tissue ECM, offering the structural support needed for cell attachment and tissue development [3, 14, 15, 16, 17, 18]. While the native ECM is considered the ideal scaffold, its complex and dynamic nature is challenging to reproduce. Tissue engineering scaffolds, therefore, aim to emulate specific ECM functions, focusing on structural, mechanical, and biochemical features to enable tissue formation [18]. The design and functionality of scaffolds are driven by their ability to influence and support cellular behaviour. Scaffolds provide structural integrity and mechanical properties tailored to the target tissue, while porous architectures often micro- or macro-porous facilitate cell migration, interaction, and ECM deposition [3]. Additionally, scaffolds act as reservoirs for bioactive molecules, including growth factors, enhancing their bioavailability and regulating cellular activities [14].

Historically, scaffold-based tissue engineering began in the mid-1980s when researchers first fabricated structures tailored to support cells with compatible physical and chemical properties [16]. Since then, the field has evolved, incorporating diverse experimental approaches to meet the functional requirements of various specialized tissues [16]. Rather than a one-size-fits-all approach, scaffold design must be multifaceted, addressing biological compatibility alongside mechanical and

architectural needs. In vivo, scaffolds extend their role beyond cellular support by providing mechanical reinforcement to damaged tissues while promoting new tissue formation. They localize cells and growth factors to target sites and create a degradable physical environment that supports neo-vascularization and tissue remodeling [18]. These characteristics make scaffolds indispensable in the development of healthy and pathological tissue models, as well as in precision medicine applications. By integrating structural, mechanical, and biochemical elements, scaffolds bridge the gap between in vitro tissue engineering and in vivo applications. Their adaptability and multifunctionality underscore their critical role in advancing tissue engineering solutions and creating innovative models for tissue repair and regeneration [17].

The native ECM serves as an ideal template for scaffold design, but its complexity and dynamic nature present significant replication challenges. Scaffolds must partially mimic ECM functions, emphasizing critical features such as architecture, bioactivity, and mechanical properties [14]. Architectural features play a critical role in scaffold functionality, scaffolds must feature high porosity and interconnected pores to support cell migration, nutrient and oxygen diffusion, and waste removal. This architecture not only facilitates cellular viability but also provides void volume for tissue formation and remodeling. Moreover, the scaffold must degrade at a rate compatible with new matrix production to ensure seamless tissue integration [14, 17, 17]. Bioactivity and biochemical cues are essential for guiding cellular behaviour. To enhance cell attachment and guide cellular behaviour, scaffolds are often modified with bioactive molecules such as RGD peptide sequences. These surface modifications improve cell-scaffold interactions without compromising structural or mechanical properties. Additionally, scaffolds can act as delivery vehicles for growth factors, enabling controlled biomolecule release for tissue regeneration [14, 16]. Mechanical properties are critical to matching the functional requirements of the target tissue. Scaffolds must closely match the mechanical properties of the target tissue to provide stability and support. Mechanobiology studies reveal that scaffold stiffness influences cell morphology, adhesion, and differentiation. For example, mesenchymal stem cells align their differentiation pathways with the stiffness of their substrate, mimicking the properties of brain, muscle, or bone tissues. This sensitivity to mechanical properties underscores the importance of tailoring scaffolds for specific applications [14, 17]. The choice of biomaterial is pivotal to scaffold performance, influencing its mechanical and degradation properties as well as its interaction with cells. For hard tissues, ceramics and their composites are valued for their stiffness and load-bearing capacity, while polymers are favored for soft tissues due to their flexibility and biocompatibility [14]. Additionally, surface properties play a critical role as the primary interface for cell attachment and behaviour modulation [17]. Advances in biomaterial selection and fabrication techniques have further enhanced scaffold functionality, enabling precise control over pore size, spatial distribution, and dynamic properties. For instance, scaffolds can incorporate time-dependent stiffening through crosslinking reactions, simulating ECM changes observed during disease progression [16].

The scope of this thesis focuses on mimicking and engineering scaffolds to replicate the ECM and studying their performance under various conditions. By tailoring scaffold properties such as architecture, bioactivity, and mechanical features, this research aims to enhance our understanding of how scaffolds interact with cells and tissues. While numerous fabrication strategies have been developed to construct such scaffolds, their applicability depends heavily on the biological context and experimental requirements. The following section provides a concise overview of the major scaffold fabrication approaches, followed by the rationale for selecting self-assembled collagen-fibronectin hydrogels in this study.

3.2.1 Overview of Scaffold Strategies

Scaffold design and fabrication significantly influence tissue regeneration by guiding cell behaviour and integration with native tissues. Four major scaffold fabrication strategies have evolved over recent decades: pre-formed porous scaffolds, decellularized ECM, cell sheets, and self-assembled hydrogels [14].

Pre-formed Porous Scaffolds are fabricated using either natural or synthetic biomaterials. Natural materials, such as ECM-derived proteins, polysaccharides, and ceramics like calcium phos-

phates, offer superior biocompatibility but often lack mechanical strength required for load-bearing applications [14, 3, 12]. Synthetic materials, including polymers and bioglasses, allow precise control over scaffold properties but often need surface modifications to enhance cell attachment and growth [17, 19]. Despite providing structural precision and wide material variety, challenges in achieving efficient and uniform cell seeding remain significant [14, 20].

Decellularized Extracellular Matrix (dECM) scaffolds utilize native ECM frameworks, stripped of cellular components, to provide a natural biochemical environment for cell seeding. dECM scaffolds support cell viability and reduce immunogenicity, yet face challenges such as mechanical weakness and incomplete cellular debris removal, which could lead to immune responses [21, 5, 22]. Issues in recellularization, including uneven cell distribution and poor vascularization, also persist [23].

Cell Sheets with Secreted ECM involve culturing cells until they form confluent layers with naturally secreted ECM, typically on thermo-responsive polymers [24, 25]. This method excels in applications requiring dense cell arrangements and rapid vascularization, such as corneal and myocardial tissues [14]. However, creating thick tissues is challenging due to practical limits in stacking multiple sheets and insufficient ECM support for load-bearing applications [14, 24].

In contrast to the previously described strategies, self-assembled hydrogels offer a more adaptable and physiologically relevant platform for cell encapsulation and ECM modeling. **Self-Assembled Hydrogels**, particularly relevant to this study, encapsulate cells in hydrophilic polymer matrices formed via stimuli-responsive polymerization methods, such as temperature-induced gelation. This category includes natural materials like collagen and Matrigel, known for their excellent biocompatibility, biodegradability, and capacity to mimic native ECM conditions [14, 26, 27]. This thesis specifically utilizes temperature-sensitive collagen–fibronectin hydrogels, which enable gentle cell encapsulation, controlled polymerization, and physiologically relevant remodeling studies [14, 28]. These hydrogels support injectable and minimally invasive applications due to their in-situ polymerization capabilities, although their mechanical properties limit them primarily to non-load-bearing applications [14]. This scaffold system was selected specifically to enable controlled gelation, real-time visualization via confocal microscopy, and investigation of fibroblast-driven ECM remodeling within a physiologically relevant 3D matrix.

Among these approaches, collagen-based hydrogels stand out, given their widespread clinical application, robust biological performance, and significant growth within the regenerative medicine market [18, 29]. Continued innovations in scaffold fabrication techniques are critical to advancing therapeutic outcomes in tissue engineering [18, 30].

3.3 Collagen–Fibronectin Hydrogel Fabrication

The fabrication technique used to produce a scaffold significantly influences its microarchitecture, mechanical properties, and biological performance. Various established methods, such as freeze-drying [14, 18, 31, 32, 33, 34, 35, 36, 37], solvent casting with particulate leaching [38, 18, 39], phase separation [18, 38, 40], electrospinning [15, 18, 19, 39, 41, 16], rapid prototyping and bioprinting [15, 18, 42], have been extensively explored for collagen-based scaffold fabrication.

Freeze-drying is a commonly used technique that creates highly porous scaffolds with interconnected pore structures closely resembling natural ECM. It preserves the bioactivity of collagen effectively; however, the mechanical strength of these scaffolds is typically limited. Additionally, freeze-drying often involves chemical crosslinking using agents like glutaraldehyde, which can potentially introduce cytotoxicity and compromise cellular compatibility [18, 31, 36].

Solvent casting combined with particulate leaching allows precise control over scaffold porosity by dissolving collagen in an appropriate solvent and adding porogen particles, which are subsequently removed to create a porous structure. Despite its simplicity and precise porosity control, this method frequently faces challenges including residual solvent contamination, incomplete removal of porogen particles, and limited mechanical robustness, restricting its suitability for certain biomedical applications [18, 38, 39].

Thermally induced phase separation (TIPS) provides control over nanoscale scaffold architecture

by phase-separating collagen solutions into polymer-rich and solvent-rich regions, followed by solvent extraction and lyophilization. Although TIPS can finely control nanostructure and porosity, it typically involves toxic organic solvents and a lengthy sublimation step, increasing complexity and production time, which can limit its practical applications [18, 38, 40].

Electrospinning generates nanofiber scaffolds that closely mimic ECM’s fibrous architecture, offering an exceptional surface area-to-volume ratio conducive to cell attachment and proliferation. However, electrospinning generally requires the use of toxic organic solvents, posing biocompatibility concerns, and the mechanical strength of electrospun scaffolds is often inadequate for load-bearing tissues without additional reinforcement strategies [15, 18, 19].

Rapid prototyping (RP) and bioprinting techniques allow high precision and reproducibility in scaffold fabrication, enabling the creation of detailed micro- and macro-structures and direct integration of living cells through bioinks. Nevertheless, RP generally produces acellular scaffolds needing subsequent cell seeding, while bioprinting faces significant hurdles in managing bioink complexity, maintaining cell viability, achieving adequate mechanical properties, and ensuring vascularization of thicker constructs [15, 18, 42]. Given these practical and biological constraints, a fabrication strategy was required that allows mild processing, supports live cell encapsulation, and enables structural tunability.

Considering the limitations of these conventional fabrication methods—such as harsh processing conditions, toxic solvents, limited mechanical robustness, and challenges in maintaining cell viability, this study employs a temperature-mediated self-assembly approach to fabricate collagen–fibronectin hydrogels under mild, physiologically relevant conditions. This technique allows for direct encapsulation of living cells with high viability, while providing tunable control over fiber formation through adjustments of gelation temperature. Consequently, these hydrogels effectively replicate the soft, hydrated microenvironment of native ECM, serving as simplified yet physiologically relevant models for investigating fibroblast behaviour, cell-driven matrix remodeling, and structure–function relationships in 3D.

The following subsections describe the specific materials, preparation protocols, and gelation conditions employed to generate reproducible collagen–fibronectin scaffolds suitable for biological and mechanical characterization.

3.3.1 Principles of Self-Assembly in Collagen-Fibronectin Hydrogels

The structural and functional properties of collagen–fibronectin hydrogels are governed by a complex set of molecular, biochemical, and physical interactions. Understanding how these interactions shape scaffold microstructure is essential for engineering reproducible and biologically relevant 3D environments for cell culture. This subsection provides a detailed theoretical background on the self-assembly of type I collagen into fibrillar networks, the environmental factors that modulate this process, and the multifaceted role of fibronectin in collagen network organization.

Special emphasis is placed on how temperature, pH, and ionic strength influence collagen fibrillogenesis, and how fibronectin modifies fiber formation and scaffold mechanics. We also distinguish between co-polymerization and adsorption as two modes of fibronectin incorporation, and examine how these microstructural outcomes impact fibroblast behaviour. These insights establish the rationale behind the temperature-controlled casting approach adopted in this study and contextualize the observed cellular responses reported in later chapters.

Molecular Mechanism of Collagen Fibrillogenesis

Type I collagen, previously introduced in Chapter 1, is a primary load-bearing ECM protein that forms triple-helical monomers known as tropocollagen. These 300 nm-long molecules spontaneously self-assemble into fibrillar structures under physiological conditions, providing the foundational architecture for fibrous tissues [43].

Under physiologically relevant conditions, typically achieved by neutralizing acidic, chilled collagen solutions and warming them to body temperature, soluble tropocollagen molecules spontaneously assemble into supramolecular fibrillar structures through a nucleation–propagation mecha-

nism [44, 45]. During this process, monomers align in quarter-staggered arrays, with the end of one molecule offset by approximately 67 nm from the next [45]. This staggered assembly gives rise to the characteristic axial periodicity known as D-banding, visible by electron microscopy as alternating light and dark bands. The 67 nm D-periodicity is a hallmark of well-organized fibrillar collagen and reflects the regular gap-overlap pattern formed by aligned tropocollagen units [44, 45].

As fibrils grow, they associate into larger fiber bundles that form a 3D collagen network, closely replicating the architecture of the native ECM. This process occurs without enzymatic mediation and is primarily driven by hydrogen bonding and ionic interactions between the telopeptide regions of adjacent collagen molecules [44]. Notably, collagen’s triple-helical structure is thermally sensitive, with a denaturation temperature around 40 °C depending on sequence and crosslinking [46]. To preserve the integrity of the triple helix and ensure proper D-banded fibril formation, self-assembly must be conducted below this thermal threshold.

Environmental Modulation: pH, Ionic Strength, and Temperature

The kinetics and morphology of collagen self-assembly are highly sensitive to the physicochemical environment. Among the most influential factors are pH, ionic strength, and temperature, each of which affects intermolecular interactions during fibrillogenesis and alters the resulting network architecture.

pH: Optimal fibrillogenesis occurs near physiological pH (7.0–7.5), where collagen molecules exhibit the highest degree of ordered self-assembly and form well-banded fibrils with consistent D-periodicity [46]. At mildly acidic pH values (e.g., 6.5), fibril formation is significantly hindered, resulting in smaller aggregates or irregular, thin fibers [47]. Conversely, mildly basic conditions (e.g., pH 8.0) may promote fibril formation but often lead to lateral packing defects or abnormal fiber morphology. Thus, a narrow pH window is critical to achieving reproducible and physiologically relevant collagen networks [43, 18].

Ionic strength: Electrolyte concentration also modulates collagen fiber formation. Physiological salt concentrations (e.g., 0.2–0.3 M NaCl or phosphate buffers) support fibril formation by screening electrostatic repulsion between monomers and stabilizing lateral aggregation. At higher ionic strengths, charge shielding can lead to tighter fiber packing but also promote sheet-like or flattened morphologies, reducing overall 3D network porosity [48, 18]. An appropriate ionic environment is essential for creating collagen gels with tunable but consistent porosity and mechanical behaviour.

Temperature: Collagen self-assembly is strongly temperature-dependent. Polymerization proceeds much faster at physiological temperatures (35–37 °C) than at room temperature or below. Lower temperatures (4–15 °C) slow the nucleation process, resulting in delayed gelation and the formation of fewer but thicker fibrils. In contrast, rapid warming promotes simultaneous nucleation events, yielding many thin fibrils and a denser, more isotropic network. However, temperatures above 40 °C risk denaturing the collagen triple helix, leading to loss of fibrillar structure [49, 50]. By carefully selecting gelation temperature, we can tune fiber thickness, pore size, and crosslinking density, key factors for modulating scaffold mechanics and cell behaviour.

Together, these environmental parameters provide a means to precisely control collagen microstructure during gel formation. Among these factors, temperature was selected as the primary tuning variable in this study due to its strong influence on fiber morphology and mechanical properties, as well as its practical controllability. The next section provides a detailed examination of how temperature-controlled casting was employed to engineer collagen–fibronectin hydrogels with defined microstructural and viscoelastic characteristics.

Fibronectin’s Role in Collagen Network Formation

Fibronectin, previously introduced in Chapter 1, is a multidomain ECM glycoprotein crucially involved in tissue development and reorganization, where it interacts extensively with cells and various matrix components. Within the specific context of collagen hydrogel formation, fibronectin exerts a complementary role by modulating fibrillogenesis kinetics, influencing the resulting hydrogel’s microstructure, and enhancing its bioactivity [51, 52]. Notably, fibronectin possesses multiple ligand-

binding domains, including a well-characterized N-terminal region that specifically interacts with collagen. This collagen-binding capability allows fibronectin to integrate directly into the collagen network during the gelation process, thereby significantly impacting fibrillogenesis and the structural organization of the resultant hydrogel [53, 54, 55].

The inclusion of fibronectin during the self-assembly of collagen hydrogels can accelerate collagen fibril formation by providing additional nucleation sites. Experimental observations from turbidity assays have demonstrated that even moderate fibronectin concentrations reduce the lag phase and amplify the rate of fibril formation, suggesting increased fibrillar mass in the final hydrogel [48, 56]. Beyond merely influencing kinetics, fibronectin also modifies the architecture of the collagen network itself. Hydrogels formed through the co-assembly of collagen and fibronectin exhibit notably heterogeneous microstructures characterized by shorter, fragmented fibrils and uneven fiber distributions compared to hydrogels composed solely of collagen. Confocal imaging and electron microscopy have consistently shown areas of clustered fibers interspersed with regions exhibiting altered pore sizes, reflecting localized and uneven polymerization events induced by fibronectin interactions [56].

Moreover, fibronectin significantly enhances the biological functionality of collagen-based scaffolds by providing additional integrin-binding domains. This expands the scope of possible cell-matrix interactions, enabling broader integrin engagement (such as integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$), which extends beyond the limited integrin interactions offered by collagen alone (mainly integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$). The availability of these diverse molecular cues is especially pertinent for fibroblast cultures, where fibronectin-dependent signaling pathways are known to critically regulate processes such as cytoskeletal organization, ECM remodeling, and cellular differentiation [57, 58].

Fibronectin integration into collagen hydrogels primarily occurs via two distinct mechanisms: co-polymerization during gelation or post-gelation adsorption onto pre-formed collagen fibrils [56]. Each approach confers unique benefits and distinctly affects scaffold architecture and mechanical characteristics. Co-polymerization, involving the pre-gelation mixing of fibronectin and collagen, results in fibronectin molecules becoming physically integrated within the collagen fibrils as they assemble. This mode of integration frequently modifies polymerization kinetics and consistently produces hydrogels with heterogeneous fibrillar structures, characterized by shorter and more fragmented fibrils with non-uniform distributions. Consequently, this structural heterogeneity introduces local stiffness gradients that can significantly influence embedded cell behaviour and matrix remodeling dynamics [56, 48].

Conversely, the post-gelation adsorption method allows fibronectin to coat pre-formed collagen fibrils without significantly altering their arrangement. Fibronectin’s high affinity for collagen ensures stable attachment and preserves the original scaffold microstructure. Comparative studies have shown that scaffolds functionalized via fibronectin adsorption maintain consistent pore sizes, fibril diameters, and mechanical properties when compared to untreated collagen gels. Adsorbed fibronectin likely localizes to specific binding sites, such as gap regions or telopeptide sites on collagen fibrils, enhancing scaffold stability by potentially providing additional crosslinking points or recruiting other ECM components. Furthermore, fibronectin remains firmly bound under physiological conditions, resisting desorption in culture media for extended periods, thereby ensuring sustained bioactivity without compromising the scaffold’s native architecture [56, 59, 60].

The choice between co-polymerization and adsorption depends significantly on experimental objectives and desired outcomes. Co-polymerization yields composite matrices comprising mixed collagen-fibronectin fibrils with enhanced bioactivity but structural heterogeneity. In contrast, adsorption maintains the collagen-dominated structure with fibronectin acting primarily as a surface bioactive cue, thus ensuring more homogeneous matrix properties. The differences in scaffold mechanics, integrin engagement, and cellular responses arising from these methods should be carefully considered when planning experimental protocols [56].

In this study, fibronectin incorporation was achieved via co-polymerization, in which fibronectin was integrated during the neutralization and gelation stages of collagen assembly. This approach was selected specifically to leverage the biological benefits of fibronectin’s direct integration, despite the accompanying structural variability. Although this choice inherently introduces fiber length variability and structural heterogeneity, it more accurately reflects the dynamic and composite nature of native ECM environments. The subsequent sections further explore the implications of this

integration strategy on scaffold organization and the cellular responses elicited by fibroblast cultures embedded within these engineered hydrogel systems.

Temperature-Controlled Casting: Microstructure and Viscoelasticity

The method of gelation, particularly the temperature profile during casting, plays a pivotal role in controlling collagen fiber morphology and network organization. Because collagen fibrillogenesis is highly temperature-sensitive, the rate at which a neutralized collagen solution is warmed from 4 °C (where collagen is soluble) to 37 °C (physiological conditions) can be tuned to influence fiber thickness, length, and pore structure.

Fast vs. slow gelation: A rapid temperature jump to 37 °C (e.g., placing the neutralized collagen solution directly into an incubator) causes collagen to nucleate and gel quickly, producing a fine fibrillar network. Rapid polymerization favors the formation of many nucleation sites simultaneously, yielding numerous thin fibers and a dense mesh with small pores. In contrast, a slower temperature increase, for example, casting the solution at 4 °C and gradually warming to 37 °C, results in fewer initial nucleation sites and provides fibrils more time to grow and laterally assemble, producing thicker fibers and more open network structures [61].

These microstructural variations translate into distinct viscoelastic properties. While some studies have reported that slowly gelled collagen networks with thicker, longer fibers may exhibit higher stiffness due to increased fibril bundling and load-bearing capacity [61], our experimental results highlight a different mechanism. Hydrogels cast at physiological temperature (37 °C) exhibited a trend toward higher storage modulus (G') compared to those formed under slower, low-temperature conditions, although this difference was not statistically significant. The denser mesh of fine, tightly entangled fibrils provided more fiber–fiber contact points per unit volume, which may have enabled more efficient elastic energy storage and resistance to deformation. In contrast, the low-density networks formed under slow warming, although composed of thicker fibrils, showed a tendency toward lower G' , likely due to fewer crosslinks and larger pores [62, 63]. These observations are consistent with reports that increased fiber density and reduced pore size are key contributors to elevated stiffness in collagen-based hydrogels [62, 63].

The apparent discrepancy with Seo et al. [61] may be explained by methodological differences. Their mechanical testing was conducted using dynamic mechanical thermal analysis (DMTA) at 10% strain—a nonlinear regime where strain stiffening and fiber alignment effects can dominate the response. In contrast, our measurements were performed using a rotational rheometer at 0.1% strain, within the linear viscoelastic range. This distinction is critical, as prior work from our group has shown that DMTA is often insensitive to subtle stiffness differences in soft collagen hydrogels.

Another important architectural feature is fiber orientation, whether the network is randomly oriented (isotropic) or aligned (anisotropic). Collagen self-assembly under typical conditions yields isotropic fiber networks unless influenced by boundary conditions or external fields. Gelation temperature can indirectly affect this by modulating the kinetics of polymerization. Slower gelation reduces convective flow and allows fibrils more time to respond to subtle directional cues. In this study, gels were cast under isotropic conditions to avoid confounding variables; however, the same temperature-mediated control could be extended to engineer anisotropic scaffolds in combination with flow or mechanical strain [64].

Our data indicate that incorporating fibronectin during self-assembly further influenced hydrogel mechanics. Fibronectin likely acts both as a biochemical ligand and as a structural enhancer by stabilizing collagen fibrils and increasing the network’s crosslinking potential. Prior studies have shown that composite hydrogels formed from collagen and fibronectin can exhibit significantly greater stiffness than collagen alone, due to fibronectin’s templating and bridging effects during fibril formation [48]. In our acellular gels, however, fibronectin’s mechanical impact was modest and consistent across gelation conditions, without producing statistically significant changes in G' .

Implications for Fibroblast–Matrix Interactions

The microstructural and mechanical properties of collagen–fibronectin hydrogels directly influence fibroblast behaviour, including cell spreading, matrix remodeling, and contractility [65]. Fibrob-

lasts are mechanosensitive cells that interpret local stiffness and pore structure through integrin-mediated adhesion and cytoskeletal tension [66]. The distinct architectures formed via temperature-controlled casting provide two mechanically and structurally different 3D microenvironments for probing cell–matrix interactions.

In fast-gelled hydrogels formed at 37 °C, the dense network of thin fibers and small pores imposes physical constraints on cell movement and initial spreading. Although these matrices exhibit higher G' and provide more integrin-binding sites per volume, the tight mesh limits the extent of cellular protrusions and hinders matrix deformation. Fibroblasts in these environments often adopt compact or stellate morphologies during early culture and require significant contractile force or proteolytic activity to initiate remodeling [67, 68].

By contrast, slow-gelled scaffolds exhibit larger pores and lower initial stiffness, creating a more permissive mechanical environment for fibroblasts to spread and exert traction forces. The increased pore space facilitates extension of cellular processes and deeper infiltration into the matrix. These gels also exhibit greater stress relaxation, which has been shown to enhance cell spreading and promote myofibroblast differentiation in soft matrices [67, 68].

Over time, fibroblasts remodel both types of matrices through matrix compaction, fiber alignment, and localized stiffening [69]. In slow-gelled scaffolds, this reorganization is especially pronounced: fibroblasts pull collagen–fibronectin fibers together, increasing fiber thickness and reducing pore size, which can significantly elevate local stiffness. Rheological measurements in cell-laden gels confirmed that fibroblasts increased G' in the slow-gelled condition more markedly than in the fast-gelled one. This feedback mechanism, where cells reshape their microenvironment and respond to evolving stiffness, mirrors native wound healing behaviour, where fibroblasts progressively transition into contractile phenotypes in response to ECM resistance [65].

These findings highlight that scaffold microarchitecture not only sets the mechanical context for fibroblasts but also determines the trajectory and extent of subsequent reorganization. The temperature-mediated casting method thus provides a controllable platform for studying cell–ECM dynamics and offers a valuable approach for engineering matrices that evolve in response to cellular activity.

3.4 Materials and Preparation Protocol

Reagents and Materials

All scaffolds in this study were fabricated using a temperature-controlled self-assembly approach based on type I collagen and fibronectin. High-concentration type I collagen (4.1 mg/mL, rat tail, Advanced BioMatrix) served as the primary matrix component. Bovine plasma fibronectin (1 mg/mL, Invitrogen, Thermo Fisher Scientific) was incorporated to modulate fibrillogenesis and provide integrin-binding domains. NIH/3T3 fibroblasts (with or without GFP expression) were used for cell-laden scaffolds.

Additional reagents included 10× phosphate-buffered saline (PBS, pH 7.4), 1 N NaOH (for neutralization), sterile distilled water (dH₂O), and culture medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin).

Neutralization and Mixing

To prevent premature gelation, all reagents were pre-chilled and mixed on ice. A neutralized collagen solution was prepared by combining collagen stock with its 10× PBS-based neutralization buffer and dH₂O in a 9:1 ratio, yielding a pH of approximately 7.4. Fibronectin was added to reach a final concentration of 0.2 mg/mL. For cell-laden scaffolds, NIH/3T3 fibroblasts were suspended directly into the collagen–fibronectin solution just before casting. The final collagen concentration in all cases was 2.88 mg/mL.

The specific casting and mixing protocols (including whether reagents were premixed or added at defined time points) varied slightly between experimental chapters and are described in detail within each results chapter where those experiments are presented.

Casting and Gelation Conditions

Scaffolds were cast into either pre-chilled standard 24-well plates or custom-made Teflon molds (dimensions detailed in subsequent chapters). The total casting volume was typically 350 μL per sample. Three gelation protocols were employed, depending on the experimental objective:

- **Fast gelation:** Samples were placed directly into a 37 °C incubator following casting, resulting in rapid fibrillogenesis and formation of fine, dense collagen networks.
- **Slow gelation (stepwise):** Gels were first held at 4 °C (15 min), then at 15 °C (10–15 min), followed by incubation at 37 °C (24 h), promoting the formation of thicker fibers with lower mesh density.
- **Room temperature gelation:** In some experiments, scaffolds were polymerized at ambient temperature (21–22 °C) for up to 15–30 min before transfer to 37 °C for full polymerization. This intermediate condition was used primarily in live-imaging studies and rheological calibration.

After 1 hour of incubation, all scaffolds were overlaid with 150 μL of complete culture medium and returned to 37 °C for equilibration prior to analysis.

Flipping Procedure

For cell-laden scaffolds, a flipping protocol was used to promote uniform cellular distribution and matrix compaction (Figure 3.1). Following the initial 1-hour incubation at 37 °C, samples were immersed in culture medium and flipped every 15 minutes for 2 hours at 27 °C. After flipping, scaffolds were returned to the incubator for an additional 21 hours of incubation. The effect of this flipping protocol on ECM structure and reorganization is examined in detail in subsequent section.



Figure 3.1: Flipping process *Schematic representation of the flipping process used to ensure homogeneous cell distribution within the collagen scaffolds.*

Sterility and Imaging Compatibility

All fabrication steps were performed under sterile conditions using a biosafety cabinet. For live-cell imaging experiments, scaffold preparation was adapted to allow casting directly into custom observation chambers under ambient conditions. This approach enabled real-time visualization of polymerization and cell–matrix interactions without disrupting the gelation process.

Fixation and Staining (for Imaging)

For structural analysis, scaffolds were fixed with 4% paraformaldehyde in PBS (10–15 min), permeabilized with 0.1% Triton X-100, and stained using Alexa Fluor Plus 488 Phalloidin (1:1000 dilution in PBS). Incubation was performed at room temperature for 20 minutes in the dark, followed by three 5-minute PBS washes to remove unbound stain. Sample-specific imaging protocols and results are detailed in Chapters 3 and 4.

3.4.1 Effect of the Flipping Method on Cell Distribution in 3D Scaffolds

In 3D hydrogel scaffolds, a known challenge is that cells tend to settle under gravity, leading to a higher concentration of cells at the bottom of the construct over time [70]. In this thesis, a flipping method was introduced to counteract this gravitational settling. These flipping experiments were conducted using collagen–fibronectin hydrogels fabricated under slow gelation conditions, where the scaffold was gradually warmed from 4 °C to 37 °C. This protocol, while beneficial for generating thick collagen fibers, also prolonged the gelation time, allowing seeded cells greater mobility prior to network solidification. As a result, gravitational settling became more pronounced than in fast-gelled scaffolds. GFP-expressing NIH/3T3 fibroblasts were suspended at a concentration of 1×10^5 cells/ml in the pre-gel solution, which was then cast into molds and incubated with or without flipping. The use of GFP-3T3 cells enabled clear visualization of cell positions via confocal microscopy and ensured consistency across all imaging experiments. The basic rationale was that by periodically inverting the cell-seeded hydrogel, unattached or loosely suspended cells would redistribute instead of all accumulating at the lowest point. This approach was intended to promote a more uniform cell density throughout the scaffold, preventing the pronounced bottom-heavy cell distribution observed with static seeding.

To evaluate how flipping affected cell distribution, we needed to visualize cells throughout the thick, hydrated scaffold. Traditional histology (e.g., embedding and sectioning) was not suitable here because the hydrogel’s high water content makes it difficult to section without distortion – dehydration can collapse the matrix, and even cryosectioning can introduce ice crystal artifacts [71]. Instead, we employed confocal laser scanning microscopy with tile scanning and z-stacks to directly image the intact hydrogel in three dimensions. Confocal microscopy is well-suited for imaging within hydrated, transparent specimens and can capture a 3D dataset without physical sectioning [72].

In practice, we first imaged the scaffold from one side (the original bottom) upward through 800 μ m of depth, then flipped the sample upside-down and repeated the z-stack from the opposite face. Capturing two opposing stacks ensured we covered the full 1.6 mm thickness of the scaffold. This strategy is important because the usable optical depth of confocal microscopy in dense tissue constructs is on the order of only a few hundred microns [73]. By imaging from both bottom and top, we mitigated signal loss in the central region and obtained a complete view of the cell distribution across the scaffold’s cross-section.

Throughout these imaging sessions, the confocal settings (such as laser power and detector gain) were kept constant for consistency between samples. One limitation we noted is that the regions with extremely high cell density – for example, the lowest part of an unflipped scaffold where many cells had settled – could cause increased light scattering and some loss of focus at deeper imaging planes. This means that in the mid-depth layers of those densely seeded, unflipped constructs, cell fluorescence was slightly under-represented due to attenuation. Nonetheless, the overall patterns of cell localization were clear, and we could reliably compare the flipped versus unflipped conditions.

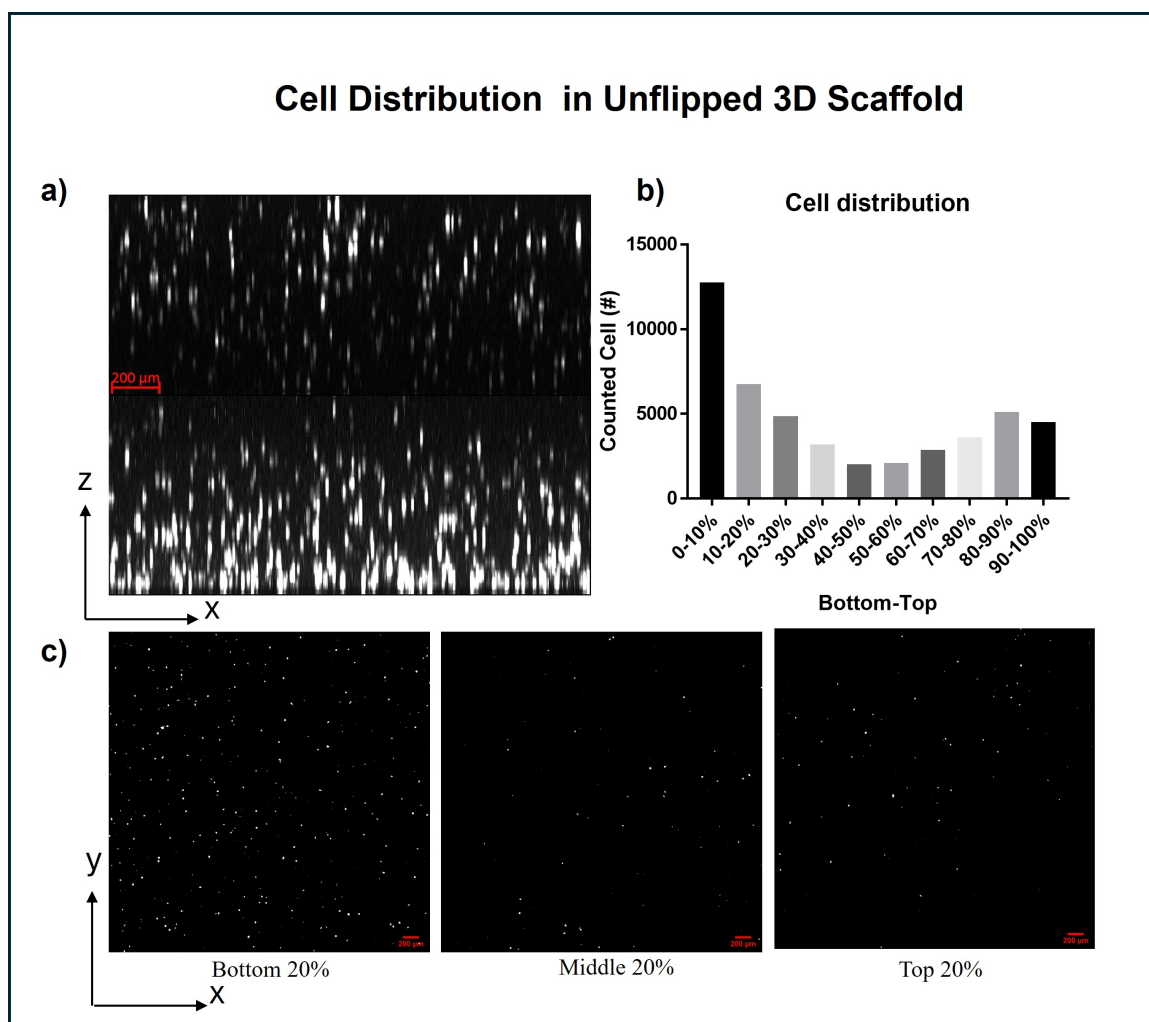


Figure 3.2: Cell Distribution Across Layers in Unflipped Scaffolds. *a) Cross view (x-z) distribution of cells from bottom to top; b) Histogram of quantitative analysis; c) Cross view (x-y) distribution of cells at different layers*

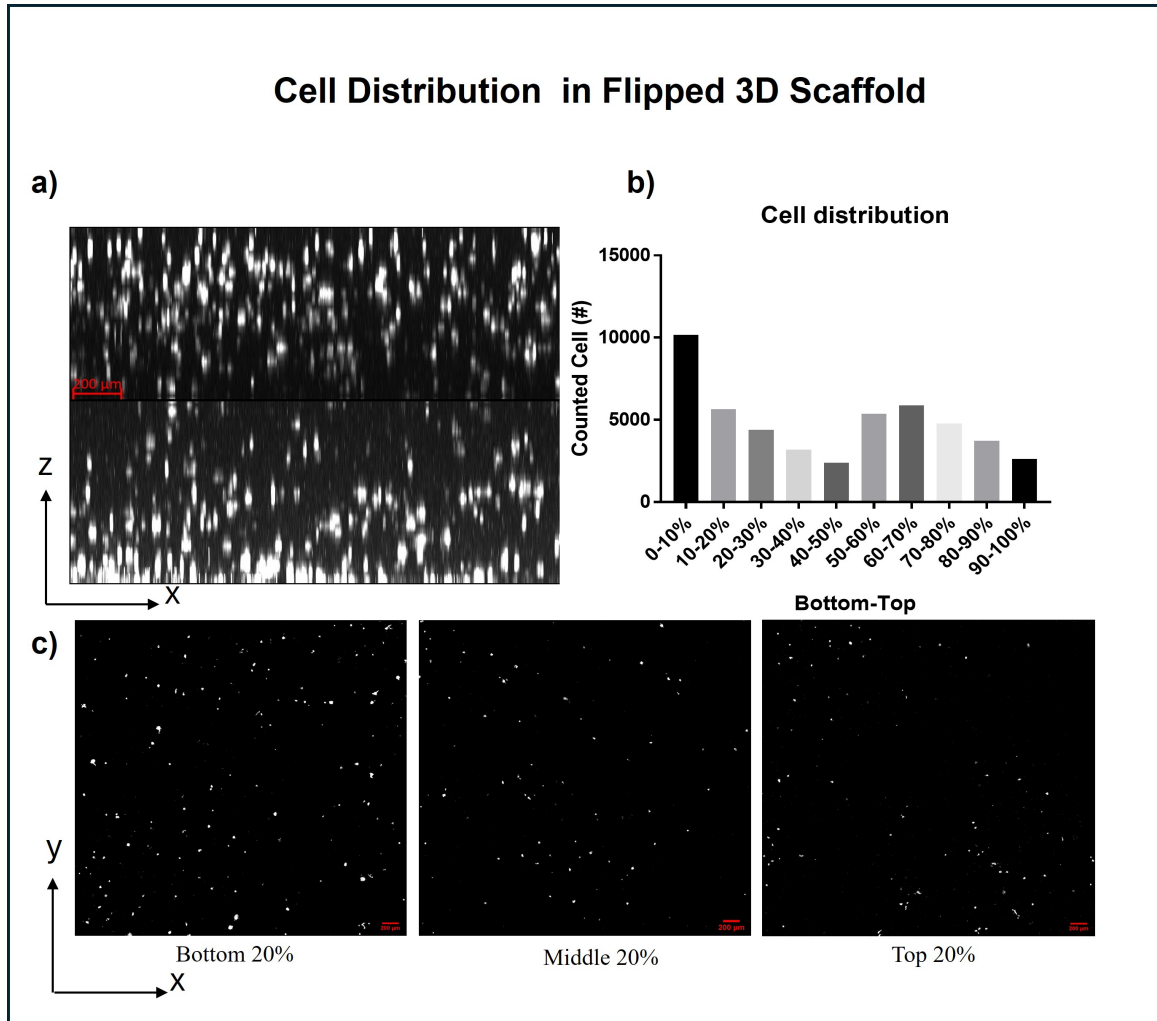


Figure 3.3: Cell Distribution Across Layers in Flipped Scaffolds. *a) Cross view (x-z) distribution of cells from bottom to top; b) Histogram of quantitative analysis; c) Cross view (x-y) distribution of cells at different layers.*

The results demonstrated that the flipping method had a marked effect on cell distribution in the 3D hydrogel (Figure 3.2, and Figure 3.3). In the unflipped control scaffolds, we observed a strong bias in cell localization toward the bottom: a dense band of cells was present at the lower surface, while the upper regions contained comparatively fewer cells. This non-uniform profile confirms that without intervention, many cells settle downward and remain concentrated at the scaffold base. In contrast, the flipped scaffolds showed a far more uniform cell distribution from bottom to top. Cells were present throughout the 800 μm imaged thickness on both sides, indicating that flipping during the seeding period successfully prevented most of the gravitational settling. Quantitative analysis (cell counts in z-sections) reflected this difference. Flipped samples had relatively even cell numbers per layer, whereas unflipped samples had an initial spike of high cell counts in the bottom-most layers and a steep drop-off toward the top. Thus, physically inverting the scaffolds allowed the previously bottom-heavy cell population to spread more evenly, yielding a balanced cell density across the cross-section.

Our findings are supported by reports in the literature and align with the general understanding of static vs. dynamic seeding. It is well documented that simple static seeding often leads to uneven cell distribution because of gravity – cells concentrate at the seeding surface or bottom of a scaffold if no mixing occurs [70]. Researchers have found that implementing dynamic seeding strategies,

which include moving the cell/scaffold system, can greatly improve uniformity. For example, one study achieved homogeneous cell attachment by periodically inverting (flipping) the scaffold during the initial seeding phase [74]. Similarly, other dynamic approaches like rotating or perfusing cell suspensions through the scaffold have been shown to provide better cell coverage throughout the scaffold than static seeding alone [73].

In our case, the simple act of flipping the construct provided that beneficial “mixing” effect without the need for specialized bioreactors. Achieving a uniform initial cell distribution is crucial, as it ensures that cells occupy the interior of the scaffold and not just the bottom, which can improve cell–cell interactions and nutrient access. Indeed, a more uniform cell seeding has been linked to better tissue outcomes; one report noted that improving the evenness of cell distribution in 3D scaffolds led to increased EXM production and mineralization compared to unevenly seeded controls [70]. Based on these results, the flipping protocol was adopted as a standard step in all subsequent cell-laden scaffold preparations to ensure uniform cellular distribution and reproducibility across experimental conditions.

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Chapter 4

Temperature-Controlled Fabrication and Characterization of 3D Collagen-Based ECM Scaffolds: The Role of Fibronectin and GFP-3T3 Cells

This chapter is based on: Javad Eslami, Parisa Sadeghi, Fan Wan, Delphine Gourdon, and James L. Harden, “GFP-3T3 Cells’ Impact on Mechanical Properties of 3D Collagen-Fibronectin Scaffolds,” to be submitted to Biomacromolecules.

4.1 Introduction

The relationship between cells and their environment is a crucial aspect of tissue development and maintenance. Cells within the ECM are surrounded by a complex network of proteins, glycoproteins, and proteoglycans, which provide structural support and biochemical signalling cues [1, 2]. In turn, cells modulate the composition and organization of the ECM, creating a dynamic feedback loop that influences tissue architecture and function [2].

Studies have shown that alterations in ECM composition and mechanics can have significant effects on cell behaviour, such as changes in adhesion, migration, proliferation, and differentiation [3, 4, 5]. For example, the stiffness of the ECM has been shown to regulate stem cell fate, with softer substrates promoting differentiation and stiffer substrates promoting self-renewal [4]. Similarly, changes in ECM protein expression and organization can alter cancer cell migration and invasion, contributing to tumour progression and metastasis [5, 6]. Therefore, understanding the relationship between the cells with the ECM and surrounding cells is crucial in biological processes. Disruptions in the balance between cells and the ECM can contribute to a variety of diseases, including fibrosis, arthritis, and cardiovascular disease [7]. For example, in pulmonary fibrosis, excessive deposition of ECM proteins such as collagen can impair lung function and contribute to respiratory failure [8]. Since any dysfunctionality of cells due to changes in ECM composition and stiffness can cause a wide range of diseases such as lung, vascular and cancer disease, the study of this relationship is important. However, the exact relationship between the ECM and cells is not clear due to the complexity of the environment. For example, cells sense and react to the dynamic forces in ECM and can change their shape and migrate to other locations; therefore, stiffness of ECMs is a factor to understand this relationship [9, 6].

On the other hand, it has been shown that cell adhesion affects cell migration, and cell adhesion highly depends on the material of the ECM [10, 8]; therefore, components of ECMs can be

considered as another factor to this relationship. Moreover, cells can reorganize the ECMs and this reorganization is dependent on many other factors, such as cell concentration, cell type, and also cell adhesion [11, 12]. Despite all that complexity, it is possible to engineer a proper condition to study these interactions in the ECM by controlling the mechanical properties of ECM at low cell concentrations.

The ECM is mainly composed of PGs and fibrous proteins. The main fibrous ECM proteins are collagens, elastin, fibronectins, and laminins which are discussed in details in the first chapter and here we briefly recall them. Collagen (Col), particularly Collagen type I (Col I), is the major component of ECM and due to its features such as biocompatibility, low toxicity, physical and immunological properties, it is a great platform for cell cultures [13]. Fibronectin (Fn) is a fibril-forming protein, whose main role is to mediate cell-matrix adhesion. Due to its flexibility and having multiple binding sites it can interact and bind to cells from multiple sites such as well-defined RGD loops, and bond to collagen fibers from the other binding sites (I_{6-9} and $II_{1,2}$) [14, 15]. It is also a ubiquitous protein in embryonic tissues and wound healing and it is needed not only in most dynamic processes of tissues like growth and wound hemostasis, but also in the progression of diseases such as fibrosis and cancer [16].

ECM mechanical properties are regulated through modifications of the microstructure and the composition of the matrix. They show viscoelastic behaviour, which is a time-dependent response of both elastic and viscous components. They usually behave anisotropically and their mechanical properties are highly sensitive to testing conditions [17]. Also, the mechanical properties of soft tissues subjected to load vary by age, pH, and the rate of applied load or deformation [18]. Moreover, changing the degree of hydration of ECM can significantly alter their viscoelasticity, which is why one needs to be extremely cautious during characterization, to maintain in native/biological conditions [19]. Among all the ECM components, studies have shown that collagen structure plays a main role in stiffness of ECM; however, crosslinkers can increase the stiffness and avoid the degradation of ECMs significantly [8, 20]. Due to this reason, many studies are focused on collagen-based ECMs. On the side of testing conditions, the stiffness of collagen-based ECMs are highly dependent on the concentration and structure of ECM which can be engineered by different methods [16].

There are several common techniques to fabricate porous scaffolds, which are discussed in chapter two in detail, including temperature-mediated casting, which is an ideal technique to engineer protein-based scaffolds due to its relatively cheap and control over scaffold architecture and an easy way of production scaffolds and was used in this study. Research on how to better replicate and modify the microarchitecture of the ECM has long been the focus of intensive study due to its robust mechanical properties. Tuneable mechanics are often a limiting factor of collagen scaffolds; these features are important in tissue engineering applications related to modelling changes in disease tissue, such as cancer, to elicit sufficient changes in cell traction forces for understanding pathologies associated with altered cell mechano-sensing.

Porous collagen scaffolds provide excellent substrates for protein adsorption, specifically Fn, and hence serve as an ECM-mimicking 3D platform to host cells for long-term in large media volumes. The interactive interplay between scaffold mechanics and presentation of Col/Fn can significantly regulate cell behaviour to create functional divergence between pathologically-relevant cell subsets. Investigating the regulation of cell response in a 3D environment with controlled mechanics, microarchitecture and Col/Fn presentation would be highly informative physiological and pathological models, in addition of being innovative when Fn conformation can also be controlled.

In this work, we investigate the effect of cells on the ECM by controlling the ECM parameters. We report the fabrication of 3D Col/Fn scaffolds with controlled mechanics, microarchitecture and protein contents prepared via an ice-templating method; these are capable of supporting 3D cell cultures in large volumes for extended culture periods (≥ 24 h). We first tuned the overall architecture and mechanics of our scaffolds. Following Col I scaffold fabrication, we used varied temperature to control Fn conformation. The thermal method we applied to control the conformation of Fn molecules adsorbed onto the Col scaffolds did not significantly affect the scaffolds bulk properties. We noticed that in presence of Fn, cells stiffen the ECM. The persistence of Fn in the presence of cells allows us to investigate cell invasion, viability, and matrix deposition within Col/Fn scaffolds with tuneable yet controlled properties. The resulting 3D scaffolds represent a versatile and powerful

new platform to control Fn conformation in tissue engineering and may pave new ways toward the development of useful tools for wound healing and mechanobiology research.

4.2 Materials and Methods

In this study, a series of collagen-based scaffolds were fabricated using the temperature-mediated casting method, with each scaffold type produced in two variations: cold cast and warm cast (Figure 4.1). The primary difference between scaffold types was the incorporation of fibronectin (Fn) and/or cells, while other factors such as mold size and material remained consistent except when intentionally varied to study their impact on ECM properties. Four scaffold types were developed: Collagen scaffolds, Collagen-Fn scaffolds, Collagen-cell scaffolds and Collagen-Fn-cell scaffolds. Additionally, the flipping method was tested as a variable influencing scaffold structure. The subsequent sections provide a detailed description of each scaffold type and the specific methodologies used in their fabrication.

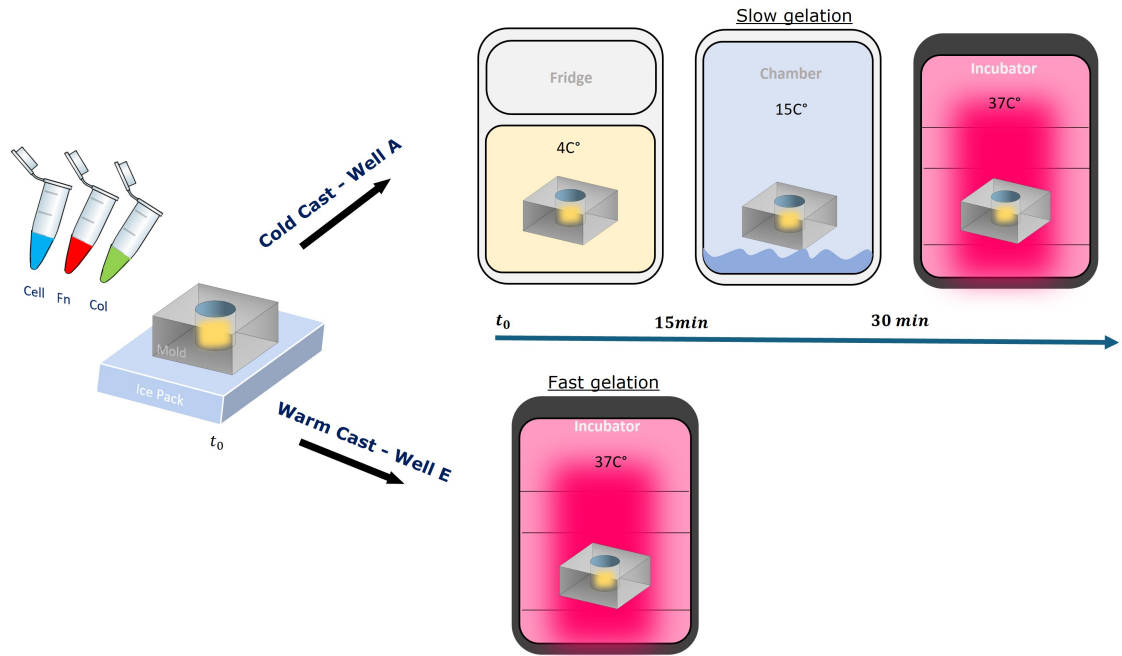


Figure 4.1: Scaffold fabrication method. Schematic representation of the temperature-mediated casting method used to fabricate collagen-based scaffolds with distinct microarchitectures. In both methods, type I collagen, fibronectin, and fibroblasts were premixed into a single homogeneous solution on ice before casting. In the cold-casting method (top), the premixed solution was added to pre-cooled molds and polymerized gradually by sequential incubation at 4°C for 15 minutes, 15°C for 15 minutes, and finally 37°C to complete gelation. In the warm-casting method (bottom), the same premixed solution was dispensed into pre-cooled molds and transferred immediately to 37°C for rapid polymerization. This temperature-controlled approach generated ECM-mimicking hydrogels with tunable fibrillar architectures.

Scaffolds were fabricated from type I collagen microfibrils derived from rat tail (*Advanced BioMatrix*, 4.1 mg/mL stock) and bovine plasma fibronectin (*Invitrogen*, *Thermo Fisher Scientific*, 1 mg/mL stock). For cell-laden scaffolds in this chapter, Green Fluorescent Protein-expressing NIH/3T3 fibroblasts (GFP-3T3) were used. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, *Sigma Aldrich*) supplemented with 10% fetal bovine serum (*Tissue Culture Biologicals*) and 1% penicillin/streptomycin (*Life Technologies*) at 37°C and 5% CO₂. An average seeding density of 1.36×10^5 cells/cm² was used to generate cell-laden scaffolds.

In all scaffold preparations, type I collagen, fibronectin, and (when applicable) fibroblasts were

premixed into a single homogeneous solution on ice prior to casting. A neutralized collagen solution was prepared by mixing collagen stock with its 10× PBS-based neutralization buffer in a 9:1 ratio, yielding a final pH of approximately 7.4. Fibronectin was then added to the neutralized collagen to reach a final concentration of 0.2 mg/mL. For cell-laden scaffolds, fibroblasts were suspended directly into the collagen–fibronectin mixture immediately before casting, ensuring uniform distribution of both fibronectin and cells throughout the pre-gel solution.

A total of 350 μ L of the premixed solution was dispensed into pre-cooled molds (0°C). For cold cast scaffolds, the filled molds were placed in a refrigerator at 4°C for 15 min, then at 15°C for 15 min in an environmental chamber, before being transferred to 37°C to complete polymerization. For warm cast scaffolds, the same premixed solution was cast into pre-cooled molds and transferred immediately to 37°C. No additional reagents were introduced into the molds after casting under either condition.

After 1 hour of polymerization at 37°C, 100 μ L of D-MEM was gently added on top of each scaffold. The medium was not mixed into the gels, as the fibrillar networks were already fully polymerized at this point. Cell-free scaffolds were then maintained in the incubator at 37°C and 5% CO₂ for a total of 24 hours from the start of polymerization.

For cell-laden scaffolds, a controlled flipping protocol was used to minimize cell sedimentation and maintain a more homogeneous cell distribution over time. Immediately after the first hour of incubation at 37°C and the addition of medium, the samples were immersed in culture medium and flipped every 15 minutes for 2 hours at approximately 27°C (room temperature). Following this flipping period, the scaffolds were returned to the incubator and maintained for an additional 21 hours at 37°C and 5% CO₂, resulting in a total incubation time of 24 hours prior to imaging and analysis.

4.2.1 Structural Characterization of Collagen Scaffold Samples Imaging

Imaging of bare Col, ColFn, and ColFn cell-laden scaffolds was quantified using a laser scanning fluorescence confocal microscope (Zeiss LSM880, Zeiss, Munich, Germany) with a 20× objective. For imaging, at least ten randomly selected regions of each sample were scanned in three dimensions (1.27 mm × 1.27 mm × 0.05 mm³, with an 8 μ m gap in the z-direction) and analyzed using Zen Blue software. In this thesis, all structural measurements and analyses presented are performed on Collagen fibers. Structural analysis of fibronectin is provided separately in the Appendix. This approach was chosen because all scaffold samples investigated here are collagen-based, and the structural organization of fibronectin was observed to closely follow and conform to the underlying collagen fiber architecture.

Although scaffolds were imaged as full 3D confocal z-stacks, pore size was quantified from the high-resolution x–y planes rather than from reconstructed 3D pore volumes. This choice was not due to limitations in resolving individual collagen fibers, the fibrils in our scaffolds were well above the confocal resolution limit. Instead, the limitation arose from the practical and computational challenges inherent to 3D pore segmentation in soft fibrillar hydrogels. First, confocal z-stacks were acquired by sequential x–y scanning, and reconstruction of tens of optical slices into a continuous 3D volume is highly RAM-dependent and prone to interpolation artifacts, especially when attempting to segment void regions that lack sharp intensity boundaries. Second, the void space in collagen hydrogels does not consist of discrete, spherical pores but rather an interconnected mesh of irregular cavities. This makes the definition of a unique 3D "pore volume" ambiguous and difficult to segment reliably from fluorescence images. Third, the computational cost of acquiring and processing high-resolution 3D datasets for tens of pores per sample is extremely high and not practical for routine quantitative analysis.

For these reasons, quantifying pore size as a 2D projected area (μ m²) from individual optical slices is a widely used and validated approach in collagen ECM characterization. Previous studies have shown that 2D pore-area measurements often differ only marginally from 3D estimates when applied consistently across samples, and they provide a robust metric for comparing relative differences between scaffold types [21, 22]. Moreover, reporting pore size as an area rather than a single radius avoids imposing circular symmetry on pores that are frequently elliptical or irregular in shape.

Accordingly, all pore-area values reported in this thesis were obtained from high-resolution x-y images using standardized ImageJ analysis across all samples.

Due to the duration of the experiment and to prevent fiber degradation, samples were fixed by immersing them in phosphate-buffered saline (PBS) (*Life Technologies*) and formalin (*Sigma Aldrich*) for 20 min before imaging.

4.2.2 Mechanical Characterization of Scaffolds by Rheometry

The mechanical properties of the scaffolds were assessed using a parallel-plate rheometer (MCR301, Anton Paar) equipped with circular plates of 12 mm in diameter, matching the sample dimensions. Prior to testing, all samples were fully hydrated. To prevent slippage or unwanted energy dissipation at the sample-plate interface, each sample was glued to both plates using *Permabond Instant Adhesive 102* (a medium-viscosity, general-purpose adhesive). In addition, the plate surfaces were sandblasted to further enhance surface adhesion.

Samples were transferred directly from the incubator to the rheometer, one at a time, to minimize any changes in hydration or temperature. All experiments were conducted at room temperature (23–24 °C). Throughout testing, deionized (DI) water was carefully added around the perimeter of each sample (see Figure 4.2) to prevent dehydration.

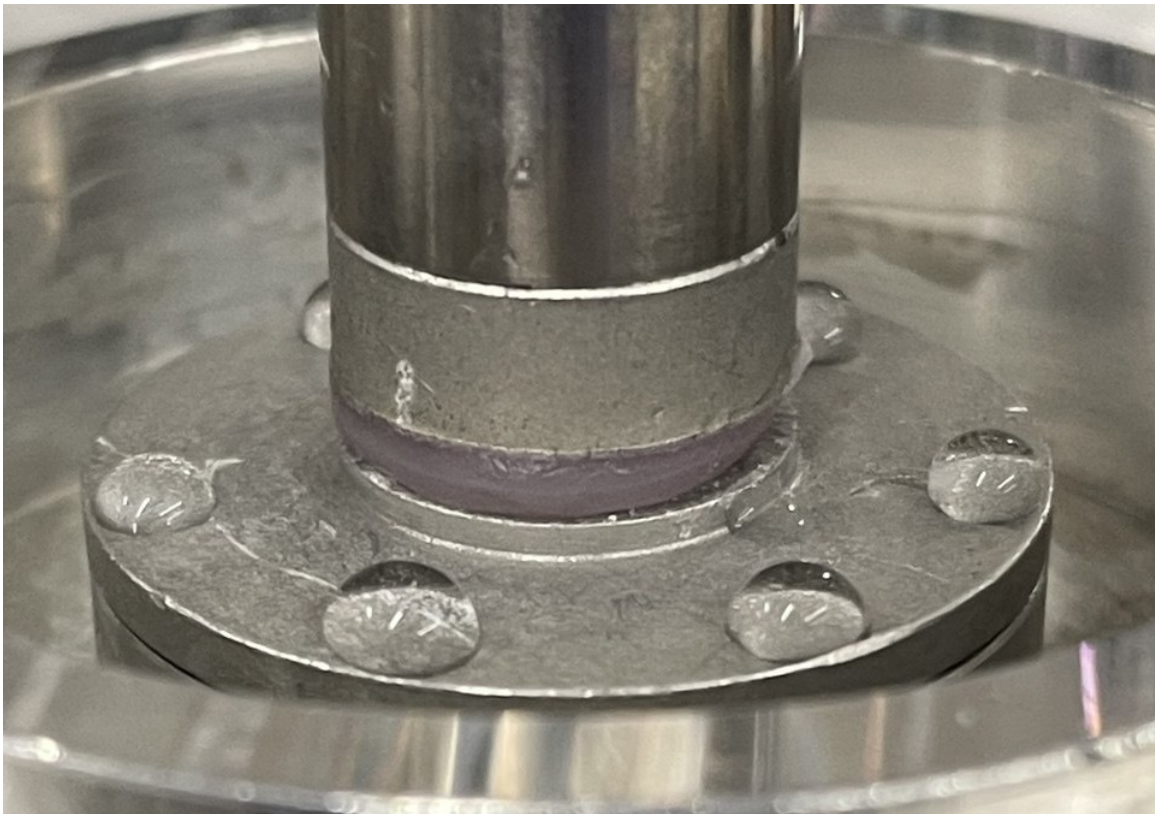


Figure 4.2: rheological measurement setup *Rheological measurement setup showing a collagen-based hydrogel sample loaded between the rheometer plates. The image captures the sample in a hydrated environment, prepared for mechanical property analysis under controlled shear conditions.*

Once mounted, each sample was subjected to small-amplitude oscillatory shear to measure its viscoelastic properties, primarily the storage modulus (G') and the loss modulus (G''). Two standard rheological protocols were used:

1. **Amplitude (Strain) Sweep.** In an amplitude sweep, the oscillation frequency ω is held constant, and the strain amplitude γ_0 is gradually increased. This test identifies the *linear*

viscoelastic region (LVR)—the range of strain amplitudes within which G' and G'' remain essentially independent of strain.

- At a fixed ω , different strain amplitudes γ_0 are applied.
 - For each γ_0 , the corresponding stress amplitude τ_0 is measured.
 - By examining how $G'(\gamma_0)$ and $G''(\gamma_0)$ vary with strain amplitude, one can determine the onset of nonlinearity and select a suitable strain amplitude within the LVR for subsequent tests.
2. **Frequency Sweep.** In a frequency sweep, the strain amplitude γ_0 is kept constant (within the LVR), while the oscillation frequency ω is varied. This reveals how G' and G'' depend on timescale, that is, how the material’s behaviour transitions between more elastic and more viscous responses with changing frequency.
- A small, fixed strain amplitude γ_0 is imposed.
 - The frequency ω is increased incrementally, and the resulting stress $\tau(t)$ is measured.
 - From the stress amplitude τ_0 between stress and strain, $G'(\omega)$ and $G''(\omega)$ are calculated for each frequency.

By plotting G' and G'' as functions of either strain amplitude or frequency, one can characterize the scaffolds’ viscoelastic behaviour. However, due to the very low moduli in these soft, water-rich 3D hydrogels, accurate measurement of the loss modulus (G'') under our experimental conditions was challenging [23, 24]. In such highly hydrated systems, the high water content tends to mask the viscous response, making G'' difficult to determine reliably. Therefore, our analysis primarily focused on the storage modulus (G'), which was measured at the frequency that provided the most consistent and dependable data. We also ensured that the chosen strain amplitude was well within the LVR to maintain linear viscoelastic conditions. For each scaffold type, at least five replicates were tested to ensure statistical robustness. Data analysis and plotting were performed using custom Python scripts.

To confirm the linear regime prior to all rheological measurements, amplitude (strain) sweeps were conducted for each scaffold type. The storage modulus G' remained constant up to approximately 0.6% strain for all samples, confirming a well-defined linear viscoelastic region (LVR). Based on these results, a strain amplitude of 0.1% was selected for all subsequent frequency sweep and time-dependent tests to ensure measurements remained within the linear regime.

For additional details, including representative amplitude sweep curves and frequency sweep plots for all scaffold types, please refer to Appendix A.

4.2.3 Statistical Analysis

Statistical analyses were performed using one-way ANOVA with Tukey’s post hoc test and Student’s t-test to determine statistical significance between conditions, using GraphPad Prism. In all cases, $p < 0.05$ is indicated by a single star (*), $p < 0.01$ by a double star (**), and $p < 0.001$ by three stars (***).

4.3 Results and Discussion

4.3.1 Baseline Scaffolds and Structural Differences

Temperature plays a crucial role in collagen polymerization, directly shaping the microarchitecture of the resulting ECM [25, 26]. While previous studies have shown that gelation temperature affects collagen fiber morphology, achieving precise control over fiber dimensions within a tunable, reproducible range remains a challenge [27]. This difficulty arises because collagen fibrillogenesis is highly sensitive to multiple environmental factors, including pH, ionic strength, and temperature, making it challenging to finely tune fiber morphology within specific intermediate thickness ranges.

In this study, we established a temperature-mediated casting approach that enables the fabrication of scaffolds with precisely modulated and repeatable microstructural differences, addressing one of the key limitation in collagen-based scaffold engineering.

The fluorescence confocal reflectance images in Figure 4.3 confirm that temperature driven polymerization produces consistent, tunable changes in scaffold architecture. The collagen scaffold's initial microstructure was strongly influenced by the casting (polymerization) conditions. Cold cast (CC) scaffolds, polymerized slowly at lower temperature, formed a coarser network with thicker, longer fibers and larger pores than warm cast (WC) scaffolds, and these trends being highly consistent across multiple samples. For example, acellular cold cast collagen had an average fiber diameter of about $3.4 \pm 0.35 \mu\text{m}$, significantly larger than the $2.3 \pm 0.25 \mu\text{m}$ fibers in warm cast gels. Fiber length followed a similar trend, with cold cast fibers reaching $52.9 \pm 8.1 \mu\text{m}$ on average compared to $38.2 \pm 4.5 \mu\text{m}$ in warm cast matrices. The more extended fibers in the slow-polymerized condition created larger void spaces (mean pore area $2541 \pm 1424 \mu\text{m}^2$ in CC vs $1798 \pm 1034 \mu\text{m}^2$ in WC). These differences are consistent with the known kinetics of collagen fibrillogenesis: a prolonged nucleation phase at low temperature allows collagen monomers to assemble into thicker fibrils before gelation completes [28, 29].

In contrast, rapid polymerization at 37°C (WC) accelerates fibril formation, yielding a denser network of thinner fibers and smaller pores. Thus, even before introducing cells, the scaffold's fiber architecture can be tuned by fabrication conditions, with cold casting producing a more open, large-fiber mesh and warm casting a finer microstructure. The ability to fine-tune fiber morphology in a controlled manner, rather than simply generating distinct extremes, is a significant advancement in scaffold engineering.

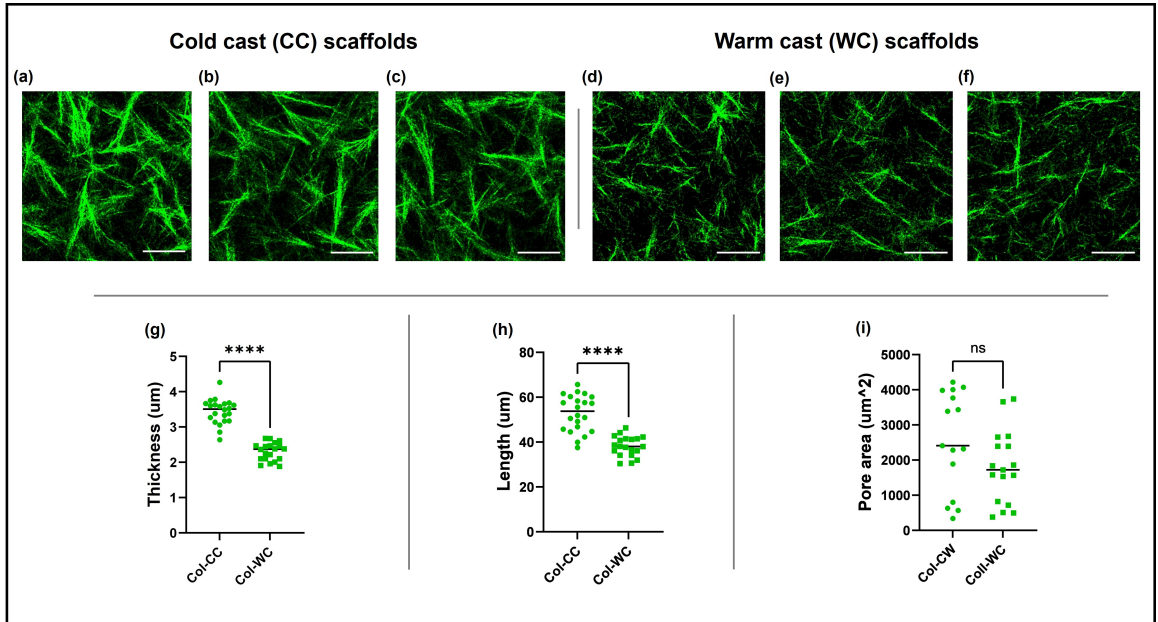


Figure 4.3: Microstructure analysis of collagen scaffolds. Combined reflectance and conventional fluorescence confocal images (Green: Collagen) of scaffolds engineered at varied polymerization temperatures: (a, b, c) Cold-cast scaffolds of Collagen from three different samples; (d, e, f) Warm-cast scaffolds of Collagen cells. Fiber topology and scaffold mesh size: (g) Average collagen fiber diameter for Collagen-Cold cast ($3.4 \pm 0.35 \mu\text{m}$), Collagen-Warm cast ($2.3 \pm 0.25 \mu\text{m}$). (h) Average collagen fiber length for Collagen-Cold cast ($52.86 \pm 8.1 \mu\text{m}$) and Collagen-Warm cast ($38.17 \pm 4.46 \mu\text{m}$). (i) Average collagen pore/mesh size for Collagen-Cold cast ($2541.6 \pm 894.34 \mu\text{m}^2$) and Collagen-Warm cast ($1797.63 \pm 1034.40 \mu\text{m}^2$). For all scaffold types, ($n \geq 15$) (Mean $\pm$ SD). Scale bars = $50 \mu\text{m}$.

An essential aspect of scaffold fabrication is controlling ECM porosity, which directly affects cell migration, nutrient diffusion, mechanical stiffness, and overall stability [25, 30]. While our

findings confirm that fiber morphology is highly tunable through temperature control, the overall scaffold porosity exhibits greater variability, particularly in cold cast scaffolds. Quantitative analysis suggests that while both warm cast and cold cast scaffolds contain a distribution of small, medium, and large pores, the cold cast scaffolds cover a broader range, indicating increased sensitivity to polymerization conditions. This suggests that while temperature control enables reproducible tuning of fiber dimensions, porosity remains more challenging to precisely regulate. The ability to modulate fiber thickness and length while still maintaining a porous structure provides a valuable degree of control for scaffold optimization; however, the observed variability in pore size highlights the inherent sensitivity of ECM architecture to polymerization conditions. Understanding these sensitivities is crucial for optimizing scaffold fabrication for specific biological applications.

The success of this temperature-controlled fabrication approach aligns with findings from previous studies [26, 31], which report similar trends in polymerization kinetics affecting fibril formation. Beyond the ability to fabricate structurally distinct scaffolds, the ability to repeatedly generate highly similar ECM architectures is crucial for understanding the sensitivity of the ECM to other biochemical and biophysical parameters. When scaffold formation is inconsistent, it is difficult to isolate the effects of additional variables such as cell interactions, crosslinkers, or other ECM components like fibronectin. By establishing a fabrication method that produces consistent results, we gain a clearer insight into how these additional factors influence ECM properties, allowing for systematic investigations into scaffold-cell interactions and ECM modifications. This not only enhances our fundamental understanding of ECM behaviour but also provides a reliable platform for optimizing scaffolds for diverse biomedical applications. The following sections will explore how this controlled fabrication method enables a deeper analysis of fibronectin integration, cellular interactions, and further scaffold modifications.

4.3.2 Biological Integration: Cell-Seeded Scaffolds

The presence of cells within the collagen matrix led to pronounced microstructural reorganization. Figure 4.4 shows that the addition of cells to collagen scaffolds in the absence of fibronectin has a significant impact on the microstructure, but not on the rheological properties of the scaffolds. Here, we study these changes more closely and discuss the possible reasons behind them.

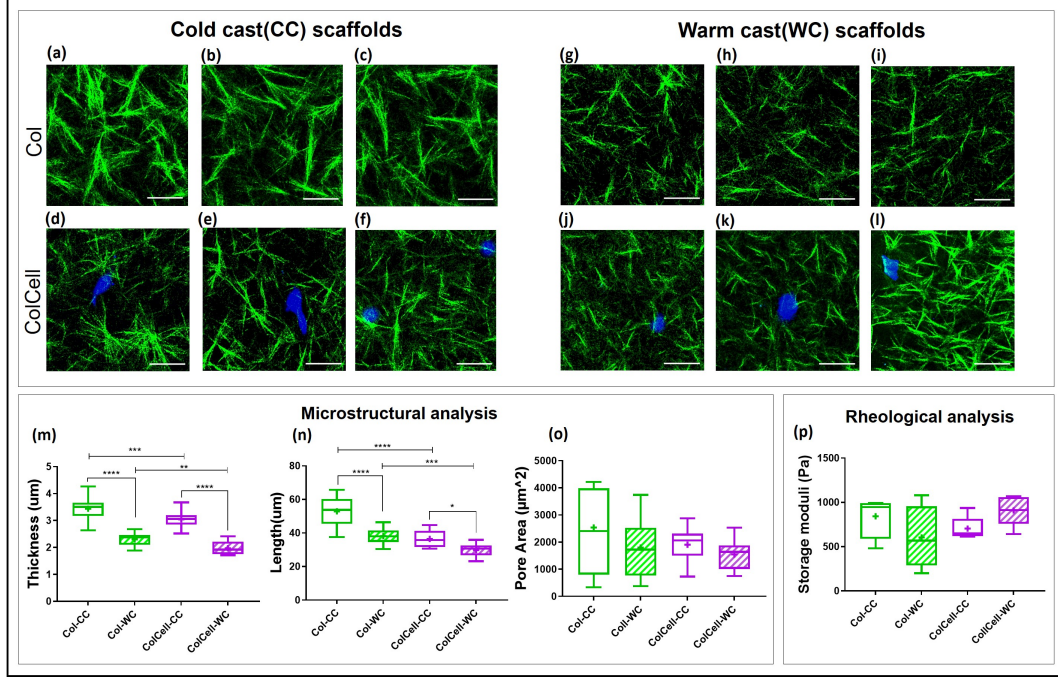


Figure 4.4: Microstructure and rheological analysis of collagen and cell-laden collagen scaffolds. (a–c) Confocal reflectance images of cold-cast collagen (Col-CC). (d–f) Cold-cast collagen–cell scaffolds (ColCell-CC). (g–i) Warm-cast collagen (Col-WC). (j–l) Warm-cast collagen–cell scaffolds (ColCell-WC). (m–o) Quantitative analysis of fiber diameter, fiber length, and pore area for all scaffold types (values summarized in Table 4.1). (p) Storage modulus G' measured at 0.1% strain and 10 s^{-1} for each scaffold type ($n=4$). Scale bar: $50 \mu\text{m}$.

Microstructural Reorganization in Cell-Laden Collagen Scaffolds

The results demonstrate that cell-seeded scaffolds exhibited shorter fiber lengths, slightly reduced fiber diameters, and smaller pores compared to their cell-free counterparts (table 4.1). In cold cast collagen with cells (ColCell-CC), the average fiber length decreased to $36.5 \pm 5.0 \mu\text{m}$ (from $52.9 \mu\text{m}$ in acellular CC), and in warm cast with cells (ColCell-WC) it dropped to $29.8 \pm 3.8 \mu\text{m}$ (versus $38.2 \mu\text{m}$ in acellular WC). This substantial shortening of fibers in the presence of cells suggests active collagen fibril degradation or reorganization by the cells. Average fiber diameter was also modestly reduced by cellular reorganization. For instance, cold cast scaffolds went from $3.4 \pm 0.35 \mu\text{m}$ (no cells) to $3.04 \pm 0.29 \mu\text{m}$ (with cells), and warm cast from $2.3 \pm 0.25 \mu\text{m}$ to $1.97 \pm 0.22 \mu\text{m}$. Concomitantly, the pore size distribution shifted toward smaller areas: cell-laden cold casts had pores around $1908 \pm 591 \mu\text{m}^2$ on average (versus $2542 \mu\text{m}^2$ without cells), and warm casts with cells averaged $1546 \pm 537 \mu\text{m}^2$ (down from $1798 \mu\text{m}^2$). Collectively, these changes indicate that embedded cells reorganize the collagen ECM into a denser meshwork with finer, shorter fibers and reduced void space. Visually, the scaffolds containing cells appeared more compact, and the quantitative image analysis confirms this densification effect. Such cell-induced microstructural shifts are in line with literature on fibroblast-populated collagen matrices – cells can pull in and rearrange collagen fibrils, aligning and tightening the network around themselves [32]. The reduction in pore size and fiber length observed in our experiments is thus a direct outcome of cells actively reorganize their surrounding matrix, transforming the initially random collagen network into a more interconnected and constrained architecture [32].

Mechanisms of Cell-Mediated ECM Reorganization

Cells achieve these modifications of the collagen matrix through several well-established mechanisms. Matrix proteolysis is an essential process in which cells release matrix metalloproteinases (MMPs),

Table 4.1: Structural and Rheological Properties of Cell-laden and Acellular Collagen Scaffolds

Scaffold Type	Fiber Diameter (μm)	Fiber Length (μm)	Pore Size (μm^2)	Dynamic Shear Moduli (Pa)
Col-CC	3.43 $\pm$ 0.35	52.9 $\pm$ 8.1	2540 $\pm$ 890	843 $\pm$ 240
Col-WC	2.30 $\pm$ 0.25	38.2 $\pm$ 4.5	1800 $\pm$ 1000	605 $\pm$ 360
ColCell-CC	3.04 $\pm$ 0.29	36.5 $\pm$ 5.0	1910 $\pm$ 590	704 $\pm$ 130
ColCell-WC	1.97 $\pm$ 0.22	29.8 $\pm$ 3.8	1550 $\pm$ 540	909 $\pm$ 170

such as collagenases, to break down collagen fibers. This targeted degradation breaks down large collagen fibrils into smaller fragments, effectively shortening fiber length and enabling the matrix to be reshaped [33, 34]. In our cell-seeded scaffolds, the significant drop in fiber length suggests collagen cleavage by cell-derived enzymes. Indeed, fibroblastic cells in collagen culture are known to produce MMPs to remodel the surrounding matrix [32]. For example, the membrane-bound MMP-14 (MT1-MMP) on fibroblasts cleaves collagen I; if this enzyme’s activity is removed, collagen accumulates unchecked and fibers remain longer and thicker [35]. Interestingly, our findings align with this. In the absence of cells and thus no active enzymatic degradation, collagen fibers stayed longer, whereas with cells (active MMP production) the fibers were truncated and network porosity affected. Collagen phagocytosis complements this process, and fibroblasts can internalize and digest collagen fragments after initial MMP cleavage, further clearing space within the matrix [36]. This turnover allows cells to progressively reorganize the scaffold, removing and replacing collagen as needed for tissue maturation.

In parallel with matrix degradation, cells also synthesize and reorganize collagen, contributing to new fibril formation (fibrillogenesis) and network stabilization. Many cells, including fibroblasts, actively deposit collagen and other ECM components into their surroundings [32]. Over time, these secreted procollagen molecules assemble into new fibrils, which can merge with or reinforce the existing scaffold. This cell-driven collagen production can partially compensate for what is degraded, but often with a different architecture. For instance, cell-deposited collagen might form finer fibrils or orient along cell surfaces [37, 38].

Moreover, cells help organize collagen fibrils via physical interactions. Fibroblasts extend dendritic processes that physically grab and reposition collagen fibers [32]. By exerting contractile forces through integrin-mediated adhesions, cells pull collagen fibrils closer together, align them along tension lines, and compact the matrix [28]. This mechanochemical process, akin to wound contraction in vivo, explains the observed reduction in pore size. The literature reports that myofibroblasts (highly contractile fibroblast derivatives) can induce significant collagen fiber alignment and densification in 3D matrices, which is consistent with our findings in cell-populated scaffolds.

As the fibers converge, new cross-links and noncovalent bonds may form between them, stabilizing the reconfigured network. Notably, cells also secrete enzymes like lysyl oxidase (LOX) that enzymatically cross-link collagen fibers. LOX-mediated crosslinking covalently bonds collagen molecules within and between fibrils, which strengthens and stiffens the ECM [39]. This crosslinking can lock in the cell-induced architectural changes, making the reorganized matrix more permanent. Overall, through a combination of proteolytic degradation, tensile force application, new matrix synthesis, and crosslinking, cells profoundly influence the collagen ECM’s microstructure.

It is important to note that LOX-mediated crosslinking was not directly measured in this study. The discussion of LOX activity is therefore intended as a possible mechanistic explanation supported by the literature rather than a confirmed process occurring in our samples. Our mechanical data cannot distinguish between biochemical crosslink formation and mechanically induced pre-tensioning of fibers, both of which can increase apparent stiffness.

Amplitude sweeps were performed on all scaffold types after the 24 h incubation period, and these confirmed that measurements were carried out within the linear viscoelastic region. However, because no rheology was performed prior to the 24 h reorganization period, these measurements cannot determine whether the critical strain or degree of pre-stress changed as a result of cell-driven reorganization. Distinguishing among these mechanisms will require future experiments such as LOX immunostaining, traction-force measurements, or nonlinear rheology of remodeled scaffolds.

Impact on ECM Mechanical Properties

As shown in figure 4.4.p, in the absence of fibronectin, the presence of cells did not significantly alter the porosity or stiffness of the ECM. While cells contributed to some degree of scaffold contraction and reorganization, these changes were not substantial enough to impact the mechanical properties of the samples. No statistically significant differences in pore area were observed between scaffold groups, with comparable average pore sizes in both cold cast (CC) and warm cast (WC) scaffolds, regardless of whether they contained cells. Similarly, the addition of cells to the collagen matrix had no meaningful effect on mean pore area, as comparisons between Col-CC vs. ColCell-CC and Col-WC vs. ColCell-WC showed no significant differences. These findings suggest that neither the polymerization condition (CC vs. WC) nor the presence of cells substantially altered the overall pore dimensions of the scaffolds.

However, the presence of cells did influence the distribution of pore sizes. Cell-laden scaffolds exhibited a narrower range of pore areas, indicating a more uniform pore size distribution compared to their cell-free counterparts. In contrast, cell-free scaffolds displayed a broader spread of pore sizes. This reduction in pore size variability suggests that cells contribute to a more homogeneous scaffold architecture, likely by filling or stabilizing larger voids, thereby constraining overall pore dimensions. While the mean pore area remained unchanged, the presence of cells appears to enhance structural uniformity within the ECM.

Although the mechanical properties of the baseline scaffolds did not change significantly, these trends provide valuable insights into how cellular interactions influence ECM organization. The cold cast scaffolds softened slightly upon cellular reorganization. The storage modulus dropped to 703.8 ± 134.2 Pa in ColCell-CC, a reduction relative to the acellular cold gel. In contrast, the warm cast scaffolds stiffened with cells – ColCell-WC had a modulus of 909.2 ± 171.8 Pa, a substantial increase compared to the cell-free warm gel. Consequently, the cell-laden warm cast matrices ultimately became even stiffer than the cell-laden cold cast ones in our experiments (approximately 909 Pa vs 704 Pa).

This intriguing reversal can be explained by the differing dominant reorganization processes. Generally, thicker fibrils present a larger surface area and may be more accessible to collagenolytic enzymes, leading to increased degradation under certain conditions [34]. However, the extent of degradation also depends on factors such as fibril cross-linking, matrix density, and enzyme diffusion [40]. In CC matrices, where fibers are thicker and relatively rigid, cell-driven degradation may become the dominant reorganization process, but the exact rate of degradation is influenced by the structural properties of the ECM and the activity of collagenolytic enzymes [34, 41]. The cells likely cleaved some of the large fibers and broke down portions of the matrix, thereby undermining the original network's integrity and reducing its stiffness. The slight compaction observed (smaller pores) was not enough to outweigh the loss of load-bearing fiber length, leading to a net softening. For instance, studies have shown that collagen degradation in arterial walls results in reduced circumferential stiffness [42, 43].

By contrast, the warm cast matrix began as a weaker, loose mesh of thin fibers, which the cells were able to compact and reinforce effectively. Here, cellular traction and matrix deposition had a larger impact [44, 45]. The cells drew the many thin fibers together, increasing fiber-fiber interactions and possibly bundling them, and they may have deposited additional matrix or induced crosslinking that fortified the network. The result was a denser fiber packing and improved connectivity, which greatly enhanced stiffness [44]. In essence, the warm cast scaffold had more room for improvement and the cells' reorganization of the matrix converted it into a much stronger structure, whereas the cold cast scaffold, initially stiff, was partly digested and reorganized in a way that slightly reduced its mechanical strength. These findings highlight that cellular reorganization can either stiffen or soften the collagen ECM, depending on the balance of matrix deposition versus degradation and the initial microstructural context.

This phenomenon is well-recognized in tissue physiology and pathology. For instance, during fibrosis, persistently activated fibroblasts deposit excessive collagen and LOX-mediated crosslinks, causing a dramatic increase in tissue stiffness [39]. Our cell-laden warm cast samples mimic this stiffening behaviour on a smaller scale, as the cells built up the matrix. Conversely, uncontrolled

proteolysis can lead to matrix softening; in extreme cases such as certain enzyme-mediated tissue breakdown, excessive collagen degradation weakens the tissue [43, 46, 47]. In our cold cast case, the cell-mediated collagen degradation and reorganization slightly tilted toward a net loss of stiffness. Studies in mice where fibroblasts lack MMP-14 (a crucial collagenase) illustrate the opposite side of the coin: without the ability to degrade collagen, fibroblasts produce abnormally dense collagen matrices that lead to increased tissue stiffness [35]. This underlines that collagen regulation is essential for maintaining proper mechanical balance – too little degradation (as in fibrosis or MMP-14 knock-out scenarios) yields an overly stiff matrix, whereas too much can erode mechanical integrity [48]. In the context of our engineered scaffolds, the cells in warm cast gels added matrix and tension (hence stiffening), whereas in cold cast gels they engaged in enough degradation to cause a slight softening. Importantly, both scenarios demonstrate that the mechanical properties of a collagen scaffold are not static but evolve due to cell activity, and understanding this evolution is crucial for designing scaffolds with desired biomechanical outcomes.

In conclusion, cells actively reorganize the composition, geometry, and mechanics of the ECM, including the collagen ECM. They synthesize ECM proteins such as collagens, particularly types I and III, to create new fibers and form crosslinks between existing fibers, which keeps the ECM in dynamic equilibrium. Meanwhile, cells apply small, fluctuating traction forces to the ECM, which deform ECM fibers, break weak bonds, and facilitate the assembly of new fibers. Therefore, cells can stiffen or soften the collagen ECM, or leave it unchanged, depending on their interaction with the collagen fibers. This interaction depends on several factors, including the density and distribution of collagen fibers, the type and expression level of crosslinkers on the cell surface, and the mechanical properties of the ECM.

Generally, an increase in the density and distribution of collagen fibers, as well as an increase in the expression level of crosslinkers, can improve the bonding strength of cells to the ECM. These findings demonstrate the complex and dynamic interplay between cells and the ECM. Understanding the factors that influence this interplay can inform the development of strategies for tissue engineering and regenerative medicine. Therefore, to fully understand the role of cells in shaping the properties of the collagen ECM, it may be necessary to incorporate other polymers, such as fibronectin, into the scaffold. By increasing the binding strength of cells to the ECM, this can allow for a greater degree of cell-mediated reorganization, which could lead to significant changes in the mechanical and other properties of the scaffold.

4.3.3 Matrix Engineering: Enhancing ECM Properties

Fibronectin’s role in modifying collagen-based hydrogels is a complex area of study and a key factor in matrix engineering, affecting scaffold microstructure, porosity, and mechanical properties. Our findings show that fibronectin incorporation influences the fibrillogenesis process, leading to structural changes in both CC and WC engineered collagen scaffolds (Figure 4.5). This section explores these effects, integrating experimental data with mechanistic insights from literature. Confocal microscopy revealed that fibronectin reduces fiber thickness, fiber length, and pore size in both CC and WC scaffolds. This trend suggests that fibronectin acts as a nucleator, accelerating the formation of smaller, more interconnected fibers [49]. Quantitative measurements confirm these effects (table 4.2).

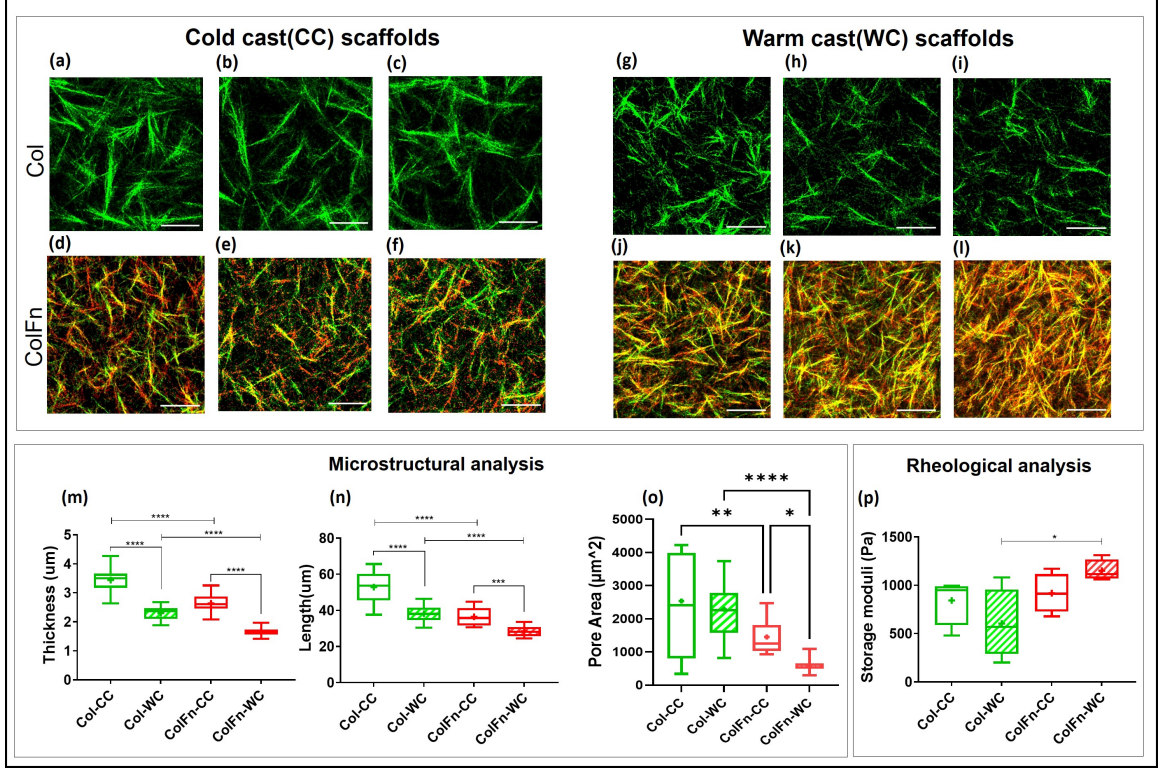


Figure 4.5: Microstructure and rheological analysis of collagen and collagen-fibronectin scaffolds. (a–c) Confocal reflectance images of cold-cast collagen (Col-CC). (d–f) Cold-cast collagen-fibronectin (ColFn-CC). (g–i) Warm-cast collagen (Col-WC). (j–l) Warm-cast collagen-fibronectin (ColFn-WC). (m–o) Quantitative analysis of fiber diameter, fiber length, and pore/mesh area for all scaffold types. (p) Storage modulus G' measured at 0.1% strain and 10 s^{-1} for each scaffold type ($n = 4$). Scale bar: $50 \mu\text{m}$.

Table 4.2: Structural and Mechanical Properties of Different Scaffold Types

Scaffold Type	Fiber Diameter (μm)	Fiber Length (μm)	Pore Size (μm^2)	Dynamic Shear Moduli (Pa)
Col-CC	3.43 ± 0.35	52.9 ± 8.1	2540 ± 890	843 ± 240
Col-WC	2.30 ± 0.25	38.2 ± 4.5	1800 ± 1000	605 ± 360
ColFn-CC	2.63 ± 0.29	36.5 ± 5.0	1450 ± 460	918 ± 180
ColFn-WC	1.66 ± 0.14	28.4 ± 3.0	593 ± 170	1150 ± 97

Fibronectin does not directly alter the molecular structure of individual collagen fibers, as demonstrated in studies using atomic force microscopy [50]. These investigations confirm that fibronectin does not modify collagen fibril mechanics at the fiber level but plays a crucial role in collagen fiber formation and organization. However, hydrogel fabrication is an inherently sensitive and dynamic process, where fiber formation is influenced by multiple external factors, including temperature and gelation kinetics. These variations led to the distinct CC and WC structures observed in our study.

By accelerating fibrillogenesis, fibronectin modifies the very conditions under which the ECM assembles, reshaping the overall scaffold architecture. Instead of allowing collagen fibers to fully extend before stabilization, fibronectin-driven nucleation results in a denser fiber network composed of thinner, shorter fibers and smaller pores. This shift occurs because the rapid fiber formation reduces the available time for elongation, leading to a more compact ECM. The structural differences between CC and WC further emphasize how fabrication conditions dictate ECM morphology with cold cast scaffolds being more structurally sensitive due to their initially larger, less interconnected fibers, and warm cast scaffolds exhibiting greater mechanical reinforcement due to their inherently smaller and denser meshwork. These findings highlight that fibronectin's effect is not just a molecular interaction with collagen but a broader modification of the scaffold fabrication process itself,

ultimately defining the structural and mechanical properties of the ECM.

Fibronectin's ability to modify scaffold fabrication conditions is a direct consequence of its interaction with collagen during assembly. As collagen and fibronectin co-polymerize, fibronectin increases the speed of fiber formation, preventing collagen from fully extending before stabilization, leading to thinner, shorter fibers and a denser ECM structure. Additionally, fibronectin interacts with collagen at specific binding sites, particularly at the 3/4–1/4 collagenase cleavage site, influencing fiber branching and stabilizing the fibrillar network [37]. The decrease in pore size observed in our scaffolds is a direct result of fibronectin enhancing fiber interconnectivity and creating a more compacted matrix. Ultimately, fibronectin alters the kinetics and spatial arrangement of collagen fibrillogenesis, leading to an ECM with reduced porosity and adjusted mechanical properties.

Although fibronectin affected both CC and WC scaffolds in similar ways, WC scaffolds exhibited greater mechanical reinforcement, whereas CC scaffolds showed more pronounced structural changes. This difference is attributed to baseline fiber properties and freezing kinetics. Cold cast scaffolds, with their larger and less connected fibers, were more structurally sensitive to fibronectin, experiencing a significant reduction in fiber length by 31% and a 53.6% decrease in pore size. In contrast, warm cast scaffolds, which inherently possess smaller and more interconnected fibers, responded differently by exhibiting a more substantial increase in shear modulus, with a 90.2% rise compared to just 9% in cold cast samples. This suggests that while CC scaffolds undergo more dramatic structural alterations, WC scaffolds exhibit a greater mechanical response. These findings align with existing literature on ECM crosslinking and fibrillogenesis, which indicate that fibronectin's effects are mechano-regulated and highly dependent on the pre-existing structural framework of the collagen scaffold [50].

Rheological analysis revealed that despite a decrease in fiber dimensions, fibronectin enhanced scaffold stiffness, as indicated by the increased storage modulus (G'). This seemingly paradoxical stiffening effect can be attributed to the enhanced fiber connectivity facilitated by fibronectin. The increased number of fiber junctions results in a denser, more percolated network, allowing stress to be more effectively distributed throughout the scaffold. Additionally, the reduction in pore size contributes to greater resistance to deformation, thereby increasing the mechanical integrity of the ECM. Although fibronectin does not form covalent bonds with collagen, it enhances non-covalent crosslinking, improving matrix elasticity and reinforcing the scaffold's mechanical properties [37].

Live imaging experiments (discussed in the next chapter) provide additional evidence that fibronectin increases the speed of fiber formation over time, reinforcing the observation that it serves as a nucleation enhancer. The ability of fibronectin to accelerate fibrillogenesis is consistent across various conditions, modifying ECM properties through its interaction with collagen during assembly.

4.3.4 Advanced Constructs: Multi-Component Systems

Microstructure and rheological analysis of cell-free collagen (ColFn) scaffolds and cell laden collagen-fibronectin scaffold (ColFnCell) is represented at figure 4.6. Based on the data, the addition of Cells to ColFn ECM had no significant effect on the scaffold's fiber thickness, length, and pore size (table 4.3). The different structure of CC and WC samples remains with almost no change (the four star significant difference between CC and WC were hold). however, comparing the data of fiber thickness, length, and pore size of ColFn-CC samples with ColFnCell-CC samples, we can see that the fiber thickness and length are the same for both types of samples, while the pore size is slightly distributed in larger range for the ColFnCell-CC samples. This result is true for the warm casting samples while the pore size is slightly smaller for the ColFnCell-WC samples. The data suggests that the presence of cells in the ColFn-CC scaffolds did not significantly affect the fiber thickness or length. However, it did result in slightly altered pore sizes. This phenomenon can be explained by the way that collagen fibers self-assemble during scaffold formation, and can be rationalized by considering the interplay between fibronectin and collagen during scaffold formation and subsequent cell interaction.

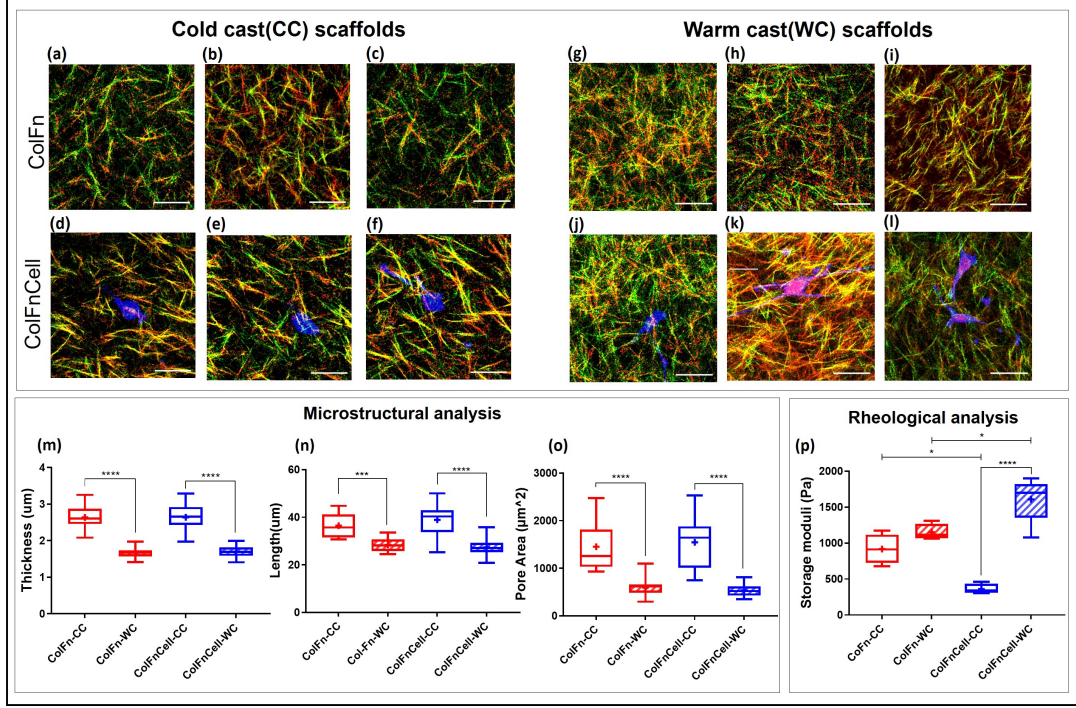


Figure 4.6: Microstructure and rheological analysis of ColFn and ColFnCell scaffolds. (a–c) Confocal reflectance/fluorescence images of cold-cast collagen–fibronectin (ColFn-CC). (d–f) Cold-cast cell-laden collagen–fibronectin (ColFnCell-CC). (g–i) Warm-cast collagen–fibronectin (ColFn-WC). (j–l) Warm-cast cell-laden collagen–fibronectin (ColFnCell-WC). (m–o) Quantification of fiber diameter, fiber length, and pore/mesh area for each scaffold type. (p) Storage modulus G' measured at 0.1% strain and 10 s^{-1} ($n = 4$). Scale bar: $50 \text{ } \mu\text{m}$.

Table 4.3: Structural and Rheological Properties of ColFn and ColFnCell Scaffolds

Scaffold Type	Fiber Diameter (μm)	Fiber Length (μm)	Pore Size (μm^2)	Dynamic Shear Moduli (Pa)
ColFn-CC	2.63 ± 0.29	36.5 ± 5.0	1450 ± 460	918 ± 176
ColFn-WC	1.66 ± 0.14	28.4 ± 3.0	593 ± 171	1150 ± 97
ColFnCell-CC	2.63 ± 0.35	38.9 ± 6.7	1546 ± 519	361 ± 61
ColFnCell-WC	1.69 ± 0.15	27.4 ± 4.1	584 ± 115	1608 ± 280

Initially, collagen-only scaffolds demonstrated sensitivity to both Fn and cellular presence, manifesting as reductions in fiber thickness, length, and porosity and as we can find from figure 4.5, and 4.4 fibronectin alters structure of ECM more than cells. However, scaffolds fabricated with both collagen and fibronectin already underwent structural changes due to the presence of fibronectin. This modification of the scaffold structure by fibronectin alters collagen fibrillogenesis, resulting in thinner, shorter fibers with reduced porosity from the outset as discussed earlier. Consequently, this defined matrix structure provides fewer opportunities for additional reorganization by cells. We acknowledge that this aspect could also be analyzed from another perspective, by directly comparing the structural changes between ColCell and ColFnCell samples to explicitly investigate fibronectin's role in presence of cells. However, since the direct effect of fibronectin on collagen ECM has already been thoroughly discussed previously, this additional analysis is provided in the appendix to maintain clarity and adhere to the primary focus of this chapter.

Furthermore, fibronectin provides robust cell adhesion sites through the RGD motif, preferentially directing cellular engagement towards fibronectin rather than collagen. This preferential adhesion, combined with fibronectin's inherent elasticity, enables fibronectin to absorb cellular mechanical forces, decreasing the necessity and extent of collagen fiber reorganization by seeded cells. In collagen-only scaffolds, cells exert significant reorganization efforts directly on collagen fibers due

to the absence of an alternative, mechanically compliant substrate like fibronectin. Thus, extensive structural alterations occur. However, when fibronectin is integrated into the scaffold matrix, its mechanical resilience and abundant cellular binding sites redirect cell interactions, mitigating further collagen remodeling.

The observed phenomenon, where the presence of cells within ColFn scaffolds leads to distinct mechanical responses in CC and WC conditions, can be better understood by examining the interplay between initial scaffold porosity, fibronectin-induced structural modifications, and subsequent cellular interactions.

In CC scaffolds, as discussed earlier, fibronectin slightly enhances initial stiffness by promoting rapid fiber nucleation, resulting in a denser, more interconnected fiber network. However, due to their inherently larger initial pore sizes and relatively less interconnected fibers, these scaffolds remain highly susceptible to cellular interactions that reduce overall stiffness [51, 47]. In this context, cells likely disrupt the ECM primarily through localized detachment or degradation of fibronectin molecules at collagen interfaces rather than direct collagen fiber degradation [52]. This disruption weakens load-bearing interactions and reduces the stiffness by decreasing effective stress transfer across the scaffold network.

In contrast, WC scaffolds inherently possess smaller, denser, and more interconnected fiber networks. This tightly interconnected architecture significantly enhances fiber-fiber interactions, which in turn significantly improves the mechanical stability of the scaffold [53]. When cells are seeded onto such a scaffold, the limited porosity restricts extensive ECM disruption. Instead, cellular forces are predominantly directed toward reinforcing the existing fibronectin–collagen network. Enhanced contractile force transmission and additional ECM deposition further strengthen scaffold stiffness and promote effective load transfer [53, 54].

Align with these explanations, recent studies demonstrate that the overall mechanical response, whether the matrix stiffens or softens, is highly dependent on how freely the matrix can contract under cell-induced forces [55]. For instance, PNIPAAm microspheres (used as cell mimics) embedded in a collagen scaffold produced contrasting effects based on boundary conditions [55]. When the matrix was mechanically constrained (limiting overall contraction), cell-like forces generated tensile pre-stress that stiffened the matrix, whereas when the matrix was free to contract, these forces induced compressive strains that led to widespread fiber buckling and ultimately softened the scaffold. This supports the idea that in our experiments, the smaller-pores (more constrained) scaffolds stiffen upon cell incorporation, while larger-pores (allowing freer contraction) soften as a result of enhanced fiber buckling.

Therefore, the differential mechanical responses observed between CC and WC scaffolds following cellular incorporation predominantly result from the initial differences in scaffold porosity and fiber interconnectivity shaped by fibronectin. While cells soften CC scaffolds primarily through localized ECM disruptions (specifically fibronectin–collagen interactions), WC scaffolds undergo additional stiffening due to enhanced fiber interconnections that resist disruptive cellular activities and favor mechanical reinforcement by cells. Thus, while larger pores may facilitate cell infiltration and nutrient diffusion, they can also render the scaffold more vulnerable to cell-mediated ECM degradation and consequent loss of mechanical integrity. Conversely, scaffolds with smaller, well-interconnected pores provide a more robust environment that limits disruptive reorganization and allows cells to actively reinforce the matrix. Balancing these factors is crucial for designing scaffolds that support both efficient tissue regeneration and long-term mechanical stability.

To further explain the underlying structural differences that drive these distinct mechanical responses and potentially influence cellular behaviour, we performed a detailed pair distribution function analysis. This structural assessment provides insights into fiber arrangement, interconnectivity, and porosity at the microscale. The PDF results allow us to directly correlate microstructural features with the scaffold’s mechanical behaviour, shedding light on how porosity and network assembly contribute to either stiffening or softening.

The Pair Distribution Function analysis

The Pair Distribution Function (PDF), also known as the radial distribution function (RDF), is a powerful statistical tool to probe the structural organization of polymer networks, including collagen-fibronectin (ColFn) scaffolds [56, 57]. This function calculates the likelihood of encountering a pair of atoms or particles at a certain distance from each other, relative to what one would anticipate from a completely random distribution.

In general terms, the PDF $g(r)$ describes how density varies as a function of distance r from a reference point (e.g., a fiber segment). When $g(r) = 1$ (in suitably normalized form), the distribution of fibers at distance r is the same as that of a completely random (ideal Poisson) distribution. Deviations from unity at various r values can indicate clustering, ordering, gaps, holes, or other non-random arrangements of fibers. In many 2D or 3D polymer networks, the PDF often shows an initial peak (short-range correlation) and eventually decays to a small baseline for large r .

Formally, for a system of N particles (or fiber segments) in a volume V (3D) or area A (2D), one common definition of $g(r)$ is:

$$g(r) = \frac{1}{\rho} \frac{dN(r)}{4\pi r^2 dr} \quad (3D) \quad (4.1)$$

or

$$g(r) = \frac{1}{\rho} \frac{dN(r)}{2\pi r dr} \quad (2D), \quad (4.2)$$

where $\rho = N/V$ (3D) or $\rho = N/A$ (2D) is the average number density, and $dN(r)$ is the number of particles in a shell (3D) or ring (2D) at distance r with thickness dr .

To improve the statistical representation of fiber distribution, we stacked adjacent layers (e.g., $n-1$, n , $n+1$) from our microscopic images. This stacking increases the sampling volume, capturing more fibers in slightly different focal planes. Subsequently, we used a manual Fiber Detection approach (see Appendix) to extract fiber coordinates and physical parameters. Although these measurements were performed in 2D slices, stacking them provides a closer approximation to the 3D network.

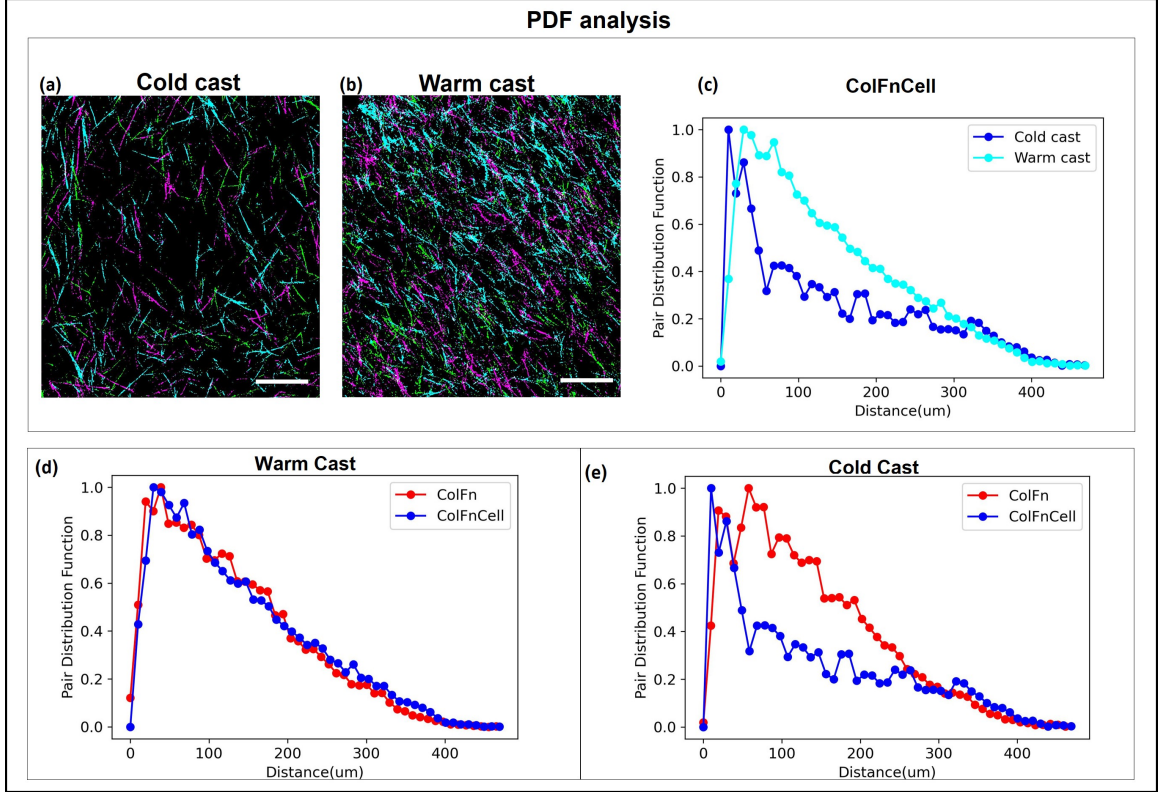


Figure 4.7: Structural analysis Stacked reflectance confocal images of collagen fibers displaying different layers (green: layer 1; cyan: layer 2; purple: layer 3). (a) Cold cast scaffold of collagen from a ColFnCell sample; (b) Warm cast scaffold of collagen from a ColFnCell sample. Scale bars = 100μm. (c) PDF of cold and warm cast ColFnCell samples; (d) PDF of warm cast ColFn and ColFnCell samples; (e) PDF of cold cast ColFn and ColFnCell samples.

Figure 4.7 shows representative PDF plots for both CC and WC, comparing scaffolds without and with cells (ColFn vs. ColFnCell). While the exact PDF values can vary from one image set to another, the general trends remain consistent. In WC scaffolds, the PDF tends to decrease more linearly with distance, indicating a distribution that is closer to random (or uniform). This suggests that fibers in WC samples do not form large localized clusters; instead, they exhibit a relatively homogeneous spacing. Consequently, when cells are present (ColFnCell-WC), they can interact with a more uniformly distributed matrix, potentially leading to different reorganization dynamics compared to CC samples. In CC scaffolds, the PDF shows a more rapid or exponential decrease, indicative of local clustering or non-uniform connectivity. In other words, once you move a short distance away from a fiber, the probability of finding another fiber drops off quickly. This can yield localized islands or denser fiber regions separated by larger pores, reducing the overall uniformity of the network.

Table 4.3 lists key structural and rheological properties for both CC and WC scaffolds, comparing pure ColFn with ColFnCell (cell-laden) variants. As seen from the Dynamic Shear Moduli, the presence of cells affects the stiffness in opposite ways depending on the casting method. ColFnCell-CC samples exhibit a notable change in storage modulus compared to ColFn-CC, which can be interpreted in light of the more clustered (exponential) PDF. Cells residing within these localized fiber clusters may reorganize or reorganize the matrix in a way that alters the mechanical response. ColFnCell-WC samples, by contrast, display a different trend, correlating with the more uniform (linear) PDF behaviour. Here, the relatively even fiber distribution may allow cells to disperse mechanical forces more evenly, affecting how the network stiffens or softens under shear.

The characteristic shapes (linear versus exponential) offer strong evidence that network architecture differs significantly between CC and WC. This difference in architecture, and in how cells

interact with it, likely underpins the observed changes in mechanical properties.

Studies on collagen-based scaffolds have long used radial distribution functions to quantify fiber-fiber spacing and network connectivity [58, 59]. A more linear PDF often correlates with relatively homogeneous networks that can distribute stresses evenly. Conversely, an exponential-like decay typically points to inhomogeneous clustering, which can lead to localized deformation and altered mechanical responses [60, 61]. In the present work, the PDF analysis supports our conclusion that the WC samples promote a more uniform fiber organization, whereas CC samples exhibit more spatially variable fiber clusters.

Future investigations could employ time-lapse confocal or multiphoton imaging to capture real-time changes in the PDF as cells reorganize the matrix. In Chapter 4, we delve further into live imaging to examine how these structural differences evolve over time under various culture conditions.

4.4 Conclusions

This chapter has demonstrated the successful fabrication and in-depth characterization of 3D collagen-based scaffolds with controlled microarchitectures using temperature-mediated casting. By employing both cold cast and warm cast techniques, we achieved distinct ECM architectures, each with unique fiber dimensions, pore sizes, and mechanical properties that closely mimic the dynamic nature of native tissues. The study confirms that scaffold microstructure can be finely tuned by adjusting polymerization conditions, with cold cast scaffolds forming thicker, longer fibers and larger pores, and warm cast scaffolds yielding a finer, denser network.

The incorporation of fibronectin into the collagen matrix further modulated fibrillogenesis by acting as a nucleation enhancer. This resulted in thinner and shorter fibers, reduced pore sizes, and, crucially, enhanced scaffold stiffness. Fibronectin also provided additional adhesion sites, which directed cell interactions away from collagen, thereby altering the cell-mediated reorganization dynamics. In scaffolds lacking fibronectin, cells reorganized the collagen matrix more directly, leading to either softening or stiffening effects that were predominantly governed by the initial microstructural context. For instance, cells softened cold cast scaffolds, likely due to more extensive local degradation, while they reinforced the warm cast networks through matrix compaction and additional ECM deposition.

Advanced image processing and pair distribution function (PDF) analysis provided quantitative insight into the spatial organization and interconnectivity of collagen fibers. The contrasting PDF behaviours (exponential decay in cold cast versus linear decay in warm cast scaffolds) corroborate the observed differences in fiber distribution and support the notion that a more homogeneous fiber arrangement in warm cast scaffolds favors uniform mechanical reinforcement by cells. Additionally, the flipping method implemented during cell seeding effectively mitigated gravitational settling, ensuring a more even cell distribution throughout the scaffold, which is vital for uniform matrix reorganization and improved nutrient diffusion.

Overall, these results underscore the intricate interplay between scaffold fabrication conditions, fibronectin-mediated structural modification, and cell-driven ECM reorganization. By delineating how cells can either stiffen or soften the ECM based on its initial microarchitecture and biochemical composition, this work provides valuable insights into tissue mechanics and mechanobiology. Such understanding is critical for the rational design of scaffolds aimed at tissue engineering, regenerative medicine, and disease modeling, where precise control over ECM properties is essential.

Future studies will benefit from real-time imaging and more extensive quantitative analyses to further elucidate the dynamic reorganization processes and to refine scaffold designs that can better replicate the complex in vivo environment. These advances will pave the way for the development of robust, biomimetic ECM platforms capable of guiding cell behaviour and promoting tissue regeneration under physiologically relevant conditions.

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Chapter 5

Kinetics of ECM Formation: Insights from Live Imaging

5.1 Introduction

In the previous chapters, we have shown that Col and Fn work together to form a crucial ECM system. This system not only gives structural support to tissues but also plays a key role in cell activities. Additionally, we demonstrated that the presence of Fn in the system led cells to actively stiffen the samples, highlighting fibronectin's importance in tissue engineering applications. Building on these foundational insights, it is essential to explore the mechanisms by which Fn integrates with the ECM and orchestrates matrix assembly. Fn contains binding domains for Col, integrins, and other ECM components, allowing it to bridge cells to the matrix and guide Col fiber formation [1]. During tissue development and repair, an initial Fn-rich matrix is assembled by cells, which then templates the subsequent deposition and alignment of Col fibers [2]. This organized assembly shows that Fn functions not only as an adhesive substrate but also as a scaffold that shapes ECM architecture from the beginning.

In addition to establishing structural scaffolding, Fn actively participates in cellular mechanotransduction and pericellular matrix remodeling [3]. Cells attach to Fn through integrin receptors, allowing them to exert contractile forces on the ECM. These forces, in turn, generate mechanical signals that are transmitted back to the cells [4]. Through these integrin–Fn connections, cells sense matrix stiffness and tension, activating pathways that regulate differentiation and matrix production [4]. Moreover, Fn fibers are sensitive to strain, meaning that tension generated by cells can stretch these fibrils, exposing hidden binding sites and changing their affinity for other proteins. This process dynamically adjusts the matrix composition. In the pericellular space around cells, Fn fibrillogenesis creates a structured scaffold that serves as a platform for other matrix molecules to assemble. Fn polymerization forms the backbone of early matrix assembly, recruiting Col and proteoglycans to create a cohesive pericellular network [5].

To elucidate these dynamic processes and capture ECM assembly and reorganization in action, advanced real-time live imaging techniques, especially confocal and multiphoton microscopy, have proven invaluable. Unlike endpoint assays, live imaging captures the spatiotemporal evolution of the matrix, revealing details about fiber number, thickness, alignment, and cell-driven reorganization that static measurements cannot. For example, confocal reflectance microscopy and time-lapse imaging have been used to directly observe Col fibers self-assembling in real time [6]. Notably, such imaging has revealed that fibril formation initiates well before bulk turbidity rises significantly, with continued turbidity growth driven primarily by fibril thickening rather than new nucleation events. This kind of insight, identifying the onset of fibril formation and distinguishing fiber number versus diameter growth, is only possible with real-time imaging. Live microscopy thus provides rich data on fiber metrics, which can complement kinetic parameters like $t_{1/2}$ and k [7, 8]. Indeed, modern optical techniques are uniquely suited for non-invasive, time-resolved monitoring of ECM assembly,

offering high spatial resolution and the ability to watch matrix architecture develop in situ [9]. Such methods have been applied to visualize Col fibrillogenesis in 3D culture, fibrin clot formation, and other matrix polymerizations, enabling researchers to directly measure fiber growth rates, branching, and network connectivity in real time [6, 10].

While real-time imaging captures the early stages of fiber formation, it also provides crucial insights into how cells dynamically reorganize the ECM over time. Time-lapse confocal microscopy of cells within collagen or fibronectin matrices has demonstrated clear feedback between cell mechanics and fiber organization. For instance, fibroblasts and other mesenchymal cells will gradually pull in and reorient collagen fibers along the axis of cell traction, a process that can be watched unfolding over hours or days. Similarly, time-lapse imaging of hepatic stellate cells embedded in 3D collagen gels revealed progressive matrix contraction and collagen fibril alignment along the axis of cellular traction forces [11]. Quantitative analysis confirmed that local fiber alignment (and increased fiber density) around each cell correlated with higher cellular contractility, supporting the idea that aligned collagen fibers serve as a fingerprint of force-generating cells [11]. This dynamic reorganization involves genuine mechanobiology. As cells exert tension, the fibrous matrix responds by realigning and stiffening in the direction of pull [11]. Live imaging has directly visualized phenomena predicted by fibrous network mechanics models: under traction, some collagen fibers buckle or break, while others align along tension lines, altering local matrix stiffness [11]. These observations highlight a matrix-cell feedback loop: cell forces can reorganize the ECM structure, and in turn the reorganized ECM (with aligned, tensed fibers) can influence cell behaviour by providing directional cues and altered mechanical resistance. Real-time imaging is crucial for capturing these feedback-driven processes, such as the formation of collagen fiber tracks that guide cell migration or the compaction of fibers between cells during tissue development [12].

In addition to mechanical influences, live imaging studies have uncovered how biochemical signals also regulate matrix reorganization. Live fluorescence microscopy has revealed that during Fn matrix assembly, deposition begins in a polarized manner at the cell’s ends, with this initial organization subsequently determining the orientation of the forming fibrils [13]. In other words, the final alignment of Fn fibers is imprinted by the spatial arrangement of early assembly events at the cell surface. By manipulating cell shape and external forces, it is possible to modulate this alignment: elongated or mechanically stretched cells tend to form more polarized and parallel Fn tracks, whereas cells on unpatterned substrates create a disordered mesh [13]. In our studies, we observed a further intriguing phenomenon: cells, under mechanical tension, generated localized Fn accumulations resembling islands or clusters.

These findings underscore how mechanical cues and biochemical factors converge to regulate ECM organization, and they were only revealed by careful live-cell imaging of the assembly process. Real-time, high-resolution microscopy of Col and Fn matrices in such experiments has enabled scientists to visualize both fiber assembly and reorganization dynamics together [10]. This approach provides a more complete picture of ECM behaviour: not only can we measure how fast fibers form (using $t_{1/2}$ and k), but we can also observe how fibers are arranged, thickened, and pulled over time in the living matrix, linking these structural changes to cellular activities and mechanical feedback. Collectively, the literature strongly supports that combining quantitative kinetic metrics with live imaging yields a powerful framework for understanding ECM assembly. It illuminates the interplay between matrix mechanics and cell function, for example, demonstrating how a cell-driven increase in fiber alignment can locally stiffen the matrix, potentially influencing neighboring cells, a fundamental concept in tissue mechanobiology [11]. Such insights are critical in fields like wound healing, fibrosis, and tissue engineering, where the dynamic evolution of the ECM (fiber deposition, alignment, cross-linking, and degradation) governs the outcome.

Despite advancements in ECM characterization, critical gaps remain in understanding the real-time dynamics of ColFn assembly and how fibroblast-mediated forces reorganize the matrix architecture in situ. Many studies rely on static or endpoint assays, missing the continuous feedback between cell-generated tension and ECM reorganization. Furthermore, the mechanisms governing localized Fn clustering and fiber orientation under mechanical load remain poorly resolved. This thesis addresses these gaps by combining live imaging with kinetic analysis to reveal the interplay between ECM assembly and fibroblast-driven reorganization.

By employing high-resolution live confocal imaging and quantitative kinetic analysis, this study offers an unprecedented view into the dynamic reciprocity between cells and the ECM. This approach not only captures the real-time kinetics of fiber formation but also reveals how mechanical cues and cell behaviours reshape the microarchitecture of the matrix, key insights that static imaging or biochemical assays cannot provide. This chapter focuses on live imaging to investigate how the ColFn ECM forms and organizes, highlighting how the presence of Fn shapes both the structural evolution of the matrix and its dynamic interactions with cells. In particular, we analyze the kinetics of fiber formation, the dynamic formation and resolution of Fn islands, and cell sedimentation behaviours within the developing hydrogel.

5.2 Materials and Methods

In this study, a series of Col, and ColFn scaffolds were fabricated to evaluate their biological and mechanical characteristics (Figure 5.1). For live imaging experiments, Col, Fn, and cells were carefully mixed under sterile conditions inside a biological safety hood, kept chilled on an ice pack to prevent premature polymerization. Due to the limitation of maintaining the experimental setup at 37°C under the microscope, the freshly prepared solution was directly injected into a custom-made mold placed under the microscope at room temperature. This approach ensured accurate observation of live cellular interactions within the scaffold despite the temperature constraint.

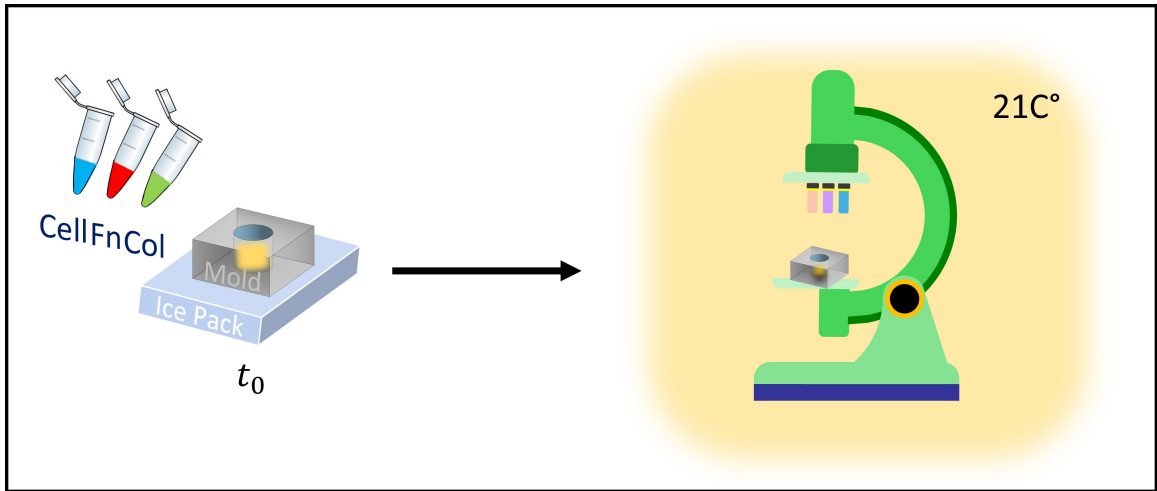


Figure 5.1: Scaffold fabrication method Schematic representation of scaffold preparation for live imaging and mechanical analysis. Collagen, fibronectin, and cells were mixed under sterile conditions on an ice pack. The scaffold mixture (collagen, fibronectin, and cells) was initially prepared on ice, then directly transferred for live microscopy imaging at room temperature (21°C).

The following sections provide detailed analyses of each scaffold type, including specific fabrication parameters, structural characteristics, and mechanical property assessments.

Structural Characterization of Collagen Scaffold Samples Imaging

For live imaging, different tile sizes were used, typically a 4×4 tile scan with a $20\times$ objective lens, to cover larger sample areas while maintaining high resolution. Z-stacks were acquired with depths ranging from $8\text{ }\mu\text{m}$ to $20\text{ }\mu\text{m}$. Time-lapse imaging was conducted for durations between 1 to 4 hours, limited by sample hydration issues. Imaging conditions were maintained at 21°C . Images were processed and analyzed using Zen Blue, with tiles stitched automatically and regions of interest selected for quantification.

5.3 Room-Temperature Kinetics of Collagen Fiber Formation

In this section, we investigate three distinct scaffold structures fabricated directly under a microscope to evaluate the formation of Col fibers over time at a fixed room temperature. We studied variations in Col polymerization at room temperature, comparing different Col concentrations and assessing the influence of added Fn. The scaffold conditions examined included two pure Col structures at concentrations of 2.88 mg/mL and 3.69 mg/mL, as well as one scaffold consisting of 2.88 mg/mL Col combined with 0.2 mg/mL Fn. The scaffold solutions were transferred directly from ice-cold conditions to room temperature, immediately injected into molds under the microscope, and imaged using z-stack tile imaging techniques. Understanding how Col concentration and Fn incorporation affect fiber formation and scaffold morphology provides valuable insights for optimizing biomaterial design in tissue engineering. Each scaffold type is discussed individually in the following subsections, accompanied by detailed analyses of observed fiber structures and their implications.

5.3.1 Collagen Scaffold Fabrication and Fiber Formation at 2.88 mg/mL

Figure 5.2 illustrates the formation kinetics of Col fibers at a concentration of 2.88 mg/mL over a 60-minute period, integrating quantitative analyses with corresponding microscopy images.

Col fibrils play a crucial role in forming extracellular networks, influencing cell behaviour, and providing mechanical support to tissues. The formation of Col fibrils, known as fibrillogenesis, is mainly driven by entropy, which is enhanced by warming and can be reversed by cooling. This process involves Col molecules assembling through both end-to-end and side-to-side interactions [14]. Initially, small groups of Col molecules form tiny fibril nuclei during the nucleation phase, resulting in a rapid increase in the number of fibers, especially within the first 12 minutes. This matches well with existing studies describing fibril assembly as beginning with nucleation and progressing into a growth phase marked by increasing turbidity [15]. Our observations similarly show a sharp increase in fiber numbers until about 12 minutes, followed by a slower increase until a stable number (approximately 39 fibers on average within a $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ area) is reached around 30 minutes.

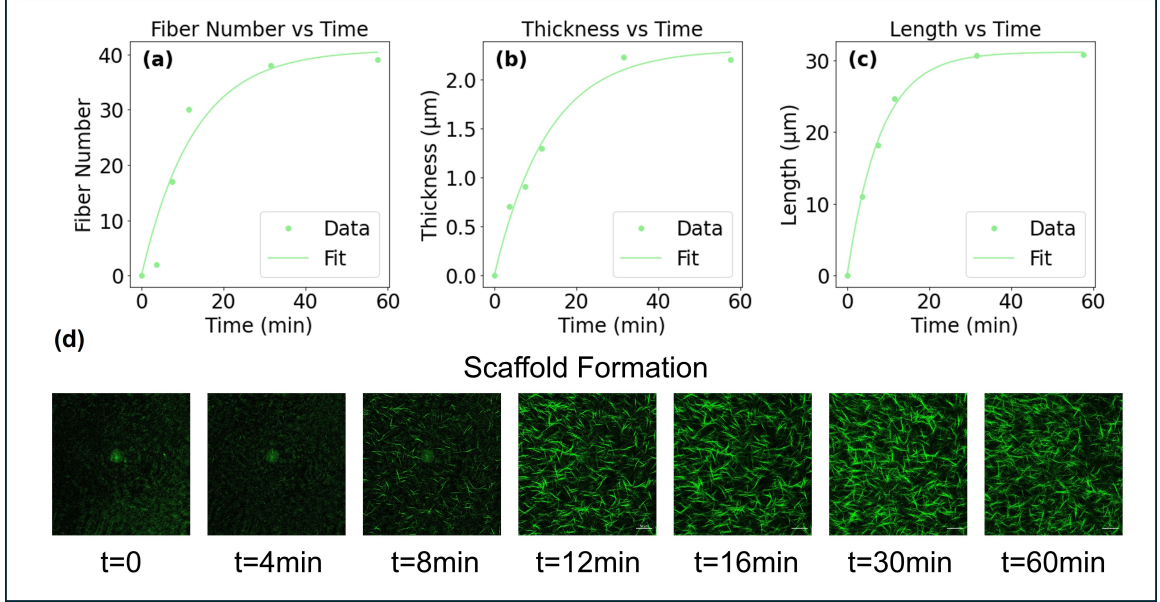


Figure 5.2: Collagen Scaffold Fabrication Kinetics. The figure demonstrates the collagen scaffold formation process at a collagen concentration of 2.88 mg/mL. (a) Number of fibers created over time. (b) Growth in fibril thickness over time. (c) Increase in fibril length over time. (d) Representative microscopy images showing morphological progression of collagen scaffold formation over a duration of 60 minutes. Scale bar: 50 μm

After the nucleation phase, fibers grow primarily by extending in length, leading to the observed stabilization in fiber numbers. At the same time, fiber thickness rapidly increases due to lateral aggregation among fibrils, stabilizing by approximately 30 minutes with an average maximum thickness of about 2.2 μm after 60 minutes. This stabilization occurs because the forces encouraging aggregation become balanced by physical limitations and constraints [16].

Fiber length also increases quickly initially as Col molecules connect end-to-end until reaching a maximum steady-state length. According to our data, fibers achieved an average length of about 30.7 μm by 30 minutes, with little change afterward. Microscopy images from time-lapse confocal reflection microscopy and Second Harmonic Generation (SHG) microscopy visually support these observations, clearly showing the transition from separate, dispersed fibrils to an interconnected, mature fiber network around 30 minutes [17].

Interestingly, even after the number of fibers became stable (around 16 minutes), some dynamic behaviour within the ECM continues. We noticed fibers occasionally appearing, disappearing, or shifting positions, suggesting active movement and interactions between fibers across different layers of the ECM. This behaviour highlights the flexibility and ongoing structural changes in Col fiber networks, which may influence their mechanical and functional properties. Altogether, these visual and quantitative findings align closely with previous research and provide deeper insights into the dynamic nature of Col scaffold formation.

5.3.2 Collagen Scaffold Fabrication and Fiber Formation at 3.69 mg/mL

The concentration of Col significantly impacts ECM properties, including fiber density, thickness, and mechanical strength. Understanding how Col fiber formation varies at different concentrations is essential for designing scaffolds tailored for specific tissue engineering applications. Figure 5.3 illustrates Col fiber formation at a concentration of 3.69 mg/mL over a 60-minute period, combining quantitative analysis with microscopy images.

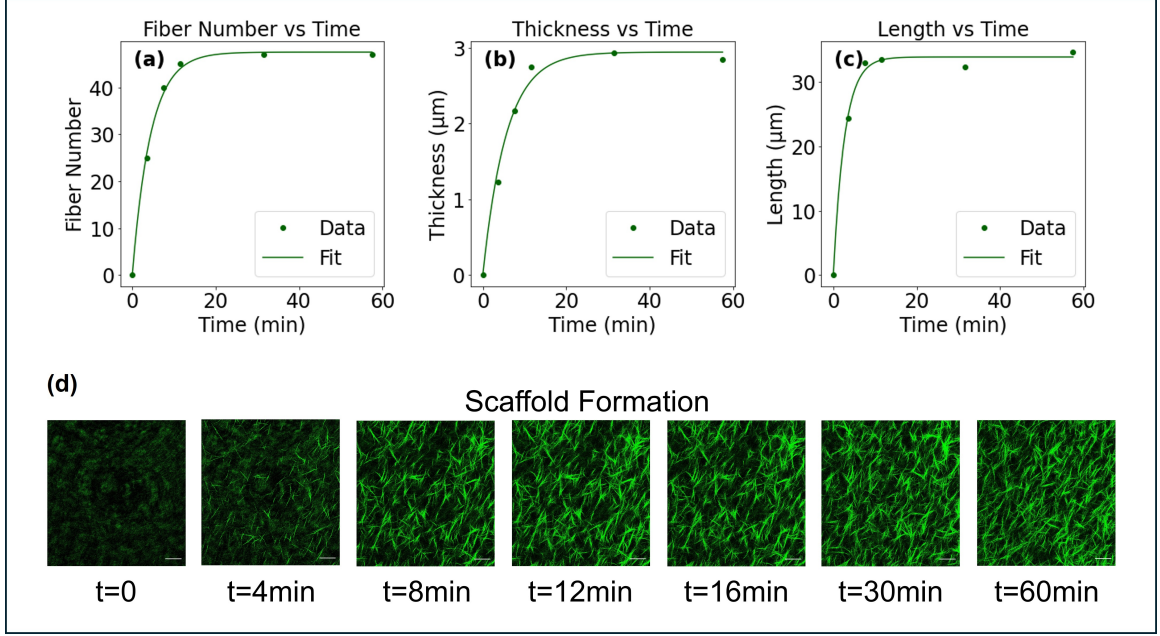


Figure 5.3: Collagen Scaffold Fabrication Kinetics. The figure demonstrates the collagen scaffold formation process at a collagen concentration of 3.69 mg/ml. (a) Number of fibers created over time. (b) Growth in fibril thickness over time. (c) Increase in fibril length over time. (d) Representative microscopy images showing morphological progression of collagen scaffold formation over a duration of 60 minutes. Scale bar: 50 μm

At this higher Col concentration, fiber formation occurs more rapidly, resulting in approximately 47 fibers within a $200 \mu\text{m} \times 200 \mu\text{m}$ area by 60 minutes. The fibers also show increased dimensions, reaching an average maximum thickness of around $2.8 \mu\text{m}$ and a length of about $34.62 \mu\text{m}$. This enhancement is primarily due to the greater availability of Col monomers, which accelerates nucleation and promotes more extensive lateral and longitudinal fibril growth. Specifically, increased Col concentrations shorten the lag phase, accelerating initial fibril formation. Additionally, higher monomer concentrations facilitate stronger lateral associations among fibrils, enhancing thickness, as well as promoting extended longitudinal growth for longer fibrils [18]. Microscopy images clearly illustrate these changes, showing a dense, interconnected scaffold structure forming by approximately 30 minutes.

It is important to consider that structural properties such as fiber thickness, length, and quantity may vary based on additional factors including collagen source, temperature, pH, ionic strength, and the presence of other extracellular matrix components. Therefore, these metrics should be interpreted within the specific experimental context [19].

The fundamental stages of Col fibrillogenesis, including nucleation and elongation, remain similar to those at lower concentrations. Nevertheless, the enhanced rate observed at higher collagen concentrations underscores concentration as a crucial factor affecting the formation speed and structural properties of collagen scaffolds. These findings highlight the importance of carefully controlling collagen concentration to develop specialized ECMs suitable for various tissue engineering applications.

5.3.3 Collagen–Fibronectin Scaffold Fabrication and Fiber Formation

Fn, another crucial ECM protein, significantly modulates cell adhesion, migration, differentiation, and overall cellular activity. Studying Col scaffold formation in the presence of Fn is particularly important because Fn serves as a key mediator between Col structures and cells, altering cell-matrix interactions and potentially affecting ECM assembly and organization. Given the substantial influence of Fn on cellular activities observed in our studies, understanding the fibrillogenesis of Col

in the presence of Fn is essential. This insight will be especially valuable as we further investigate how cells reorganize ECM structures when Fn is incorporated.

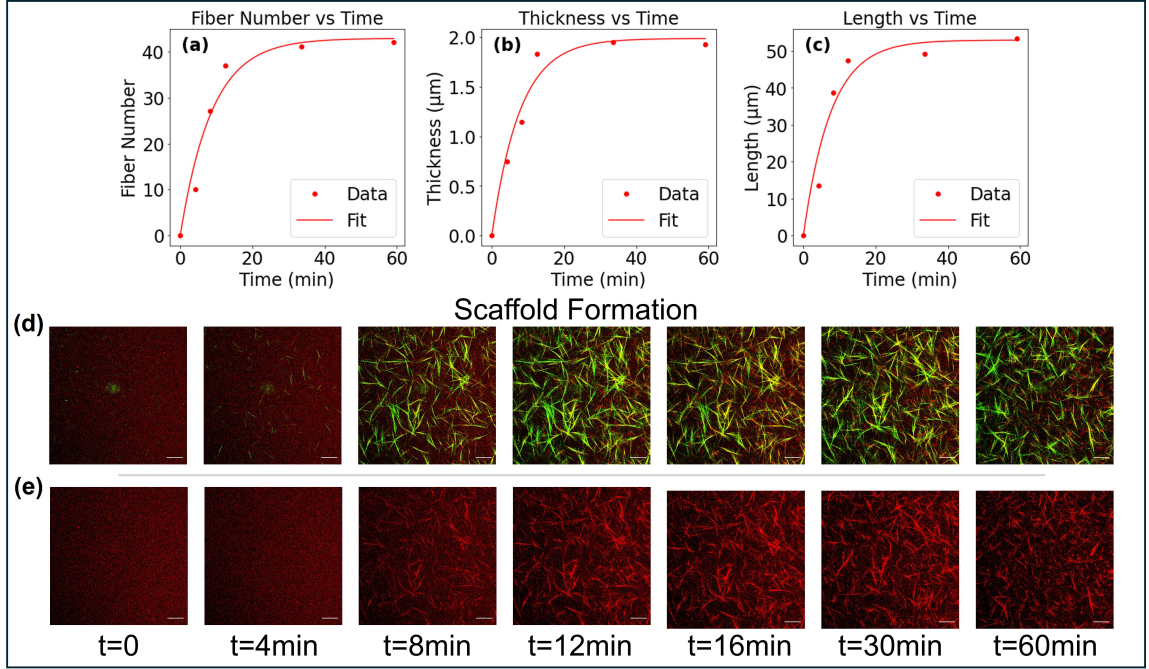


Figure 5.4: Collagen-Fibronectin Scaffold Fabrication Kinetics. This figure illustrates the scaffold formation process for collagen (Col: Green) and fibronectin (Fn: red) at concentrations of 2.88 mg/ml and 0.2 mg/ml, respectively. All measurements presented are based on collagen fibers. (a) Number of fibers formed over time. (b) Fiber thickness growth over time. (c) Increase in fiber length over time. (d) Representative microscopy images demonstrating the morphological development of the collagen scaffold over 60 minutes. (e) Representative microscopy images showing the morphological progression of fibronectin scaffold formation over 60 minutes. Scale bar: 50 μm .

Figure 5.4 shows the formation kinetics of collagen-fibronectin (ColFn) scaffolds, specifically examining the Col fiber assembly process over 60 minutes at concentrations of 2.88 mg/mL for Col and 0.2 mg/mL for Fn.

In the ColFn system, Col fiber formation initially occurs rapidly, producing approximately 42 fibers per $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ area by around 60 minutes. Fiber thickness quickly reaches a maximum average value of approximately 1.93 μm , stabilizing after about 30 minutes. Similarly, fibers grow rapidly in length, reaching an average maximum length of approximately 53.35 μm by the end of the observation period. The presence of Fn may facilitate and stabilize Col fibril interactions, leading to distinctive morphological features and kinetics compared to pure Col scaffolds. Microscopy images clearly illustrate the morphological development of Col scaffolds and demonstrate Fn's concurrent structural assembly. Moreover, we observe a lag between the formation of Fn fibers and Col fibers. Although under many physiological conditions Fn fibers form first and serve as a scaffold for Col deposition [20], in our system the low concentration of Fn relative to the high Col concentration favors rapid spontaneous collagen fibrillogenesis, leading to an earlier appearance of collagen fibers.

Given these initial observations, the detailed comparison of pure Col scaffolds at different concentrations and ColFn scaffolds, as explored in subsequent sections, will provide critical insights into how Fn influences the structural dynamics and biological functionality of ECMs. This comparative approach helps clarify the underlying mechanisms through which Fn modifies Col scaffold formation and its implications for tissue engineering applications.

5.4 Fiber Formation Kinetics Analysis

To quantitatively compare fiber formation kinetics as well as Col structural dynamics (fiber count, thickness, and length) across conditions, namely ColFn, Col at 2.88 mg/mL , and Col at 3.69 mg/mL we employed two complementary normalized metrics for each parameter (the half-maximum time, and the exponential rate constant). In all cases, normalization was necessary due to variations in the plateau (or final) values across conditions, thereby enabling direct comparisons of kinetic profiles.

Half-maximum time ($t_{1/2}$) and exponential rate constants (k) are well-established parameters for quantifying the kinetics of extracellular matrix (ECM) fiber assembly. In collagen fibrillogenesis assays, researchers routinely define $t_{1/2}$ as the time required for an assembling collagen matrix to reach half of its final turbidity or fiber density [8]. This metric provides a convenient single-value summary of assembly speed, allowing comparison of how different conditions or additives accelerate or delay fiber formation [7].

Half-maximum time ($t_{1/2}$) has long been used as a standard metric for characterizing the kinetics of collagen and other biopolymer assembly [21, 7]. It distills complex assembly dynamics into a single, quantitative parameter that reflects overall polymerization speed and can be correlated with experimental conditions. This parameter is particularly valuable for experimental planning, providing guidance on incubation times for matrix stabilization or indicating how rapidly gelation occurs under different conditions. Overall, $t_{1/2}$ is a widely accepted benchmark for comparing assembly kinetics in various extracellular matrices and self-assembling biological systems.

In parallel, kinetic analyses of matrix assembly often assume a first-order or exponential model, yielding an apparent rate constant (k) to describe assembly speed. For example, in Fn matrix assembly, the incorporation of Fn into an insoluble matrix has been shown to follow first-order kinetics [22].

The use of an exponential k in that context treated matrix assembly much like a chemical reaction, reinforcing that an ECM fiber network can form with definable reaction kinetics. Likewise, in collagen studies, researchers sometimes fit the growth phase of fibril assembly to kinetic models [23, 24].

Both $t_{1/2}$ and k provide complementary, simplifying metrics (half-life vs. rate constant) that are widely used to characterize ECM assembly dynamics for Col, Fn, fibrin, and other polymeric matrices. These metrics have become essential in mechanistic studies, such as testing the effects of pH, enzymes, cross-linkers, or mutations on fibril formation rates, as well as in engineering applications where tuning scaffold gelation times is crucial for cell encapsulation protocols [7, 21].

For any given variable $X(t)$ (where X represents the fiber count N , Col thickness T , or Col length L), the data were first normalized as

$$f_X(t) = \frac{X(t)}{X_{\text{final}}} \quad (5.1)$$

where X_{final} denotes the plateau value for that condition.

We defined $t_{1/2}$ as the time required for the normalized variable to reach 50% of its plateau value, determined via linear interpolation between adjacent time points. To distinguish among parameters, we denote these as

$$t_{1/2,N} \quad \text{for fiber counts,} \quad (5.2)$$

$$t_{1/2,T} \quad \text{for thickness,} \quad (5.3)$$

$$t_{1/2,L} \quad \text{for length.} \quad (5.4)$$

Assuming that the early phase of the normalized growth follows an exponential model, we approximated

$$f_X(t) = 1 - e^{-k_X t} \quad (5.5)$$

which relates $t_{1/2}$ to the rate constant k_X as

$$k_X = \frac{\ln(2)}{t_{1/2}} \quad (5.6)$$

where the rate constants are denoted as

$$k_N \text{ for fiber counts,} \quad (5.7)$$

$$k_T \text{ for thickness,} \quad (5.8)$$

$$k_L \text{ for length.} \quad (5.9)$$

These approaches were applied to every parameter across all experimental conditions, and the resulting data are shown in Figure 5.5, which compares Col properties at different concentrations as well as in the presence of Fn. The calculated kinetic metrics for each parameter and condition are summarized in Table 5.1 and Table 5.2.

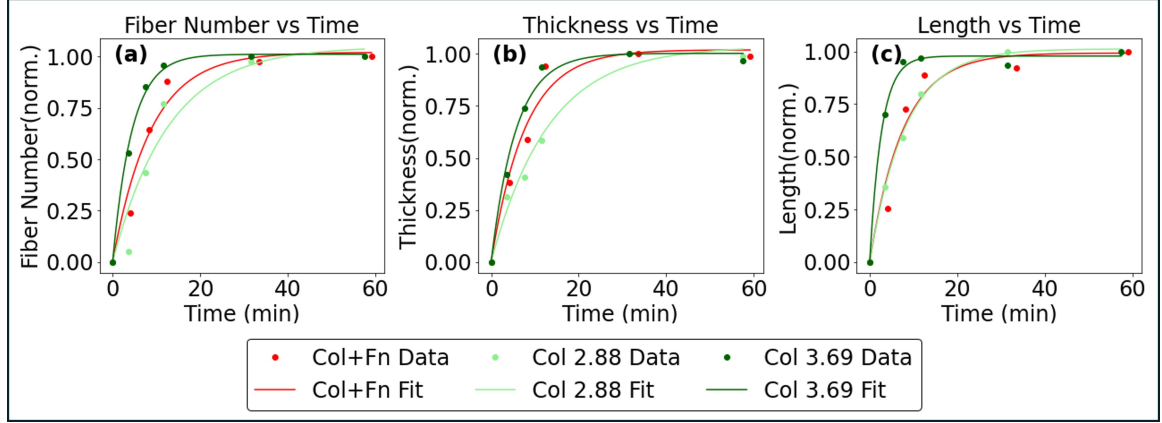


Figure 5.5: Normalized Collagen Scaffold Kinetics. This figure shows the normalized evolution of collagen scaffold parameters over time for three conditions: ColFn (red), Collagen at 2.88 mg/ml (light green), and Collagen at 3.69 mg/ml (dark green). (a) Normalized fiber number, (b) normalized fiber thickness, and (c) normalized fiber length.

Table 5.1: Half-Maximum Time Analysis

Condition	$t_{1/2,N}$ (min)	$t_{1/2,T}$ (min)	$t_{1/2,L}$ (min)
ColFn	6.62	6.50	6.29
Col (2.88 mg/ml)	8.35	9.68	6.06
Col (3.69 mg/ml)	3.37	4.59	2.55

Table 5.2: Exponential Rate Constant Analysis

Condition	k_N (min^{-1})	k_T (min^{-1})	k_L (min^{-1})
ColFn	0.105	0.107	0.110
Col (2.88 mg/ml)	0.083	0.072	0.114
Col (3.69 mg/ml)	0.206	0.151	0.272

Using collagen at 2.88 mg/mL as the baseline, increasing the Col concentration to 3.69 mg/mL significantly accelerated fiber formation. Specifically, the half-maximum time for fiber formation ($t_{1/2,N}$) decreased from 8.35 minutes at 2.88 mg/mL to 3.37 minutes at 3.69 mg/mL. Correspondingly, the exponential rate constant (k_N) more than doubled, from 0.083 min^{-1} to 0.206 min^{-1} . Moreover, the average number of fibers increased from 39 at 2.88 mg/mL to 47 at 3.69 mg/mL, indicating a denser fiber network at the higher Col concentration.

In line with these findings, incorporating Fn into Col at 2.88 mg/mL (ColFn) resulted in differences in the fiber formation kinetics: $t_{1/2,N}$ decreased from 8.35 minutes to 6.62 minutes and

k_N increased from 0.083 min^{-1} to 0.105 min^{-1} , indicating that Fn alters the formation rate at this concentration, though not as dramatically as increasing Col concentration does. Accordingly, its presence slightly modifies the scaffold structure, with the fiber number increasing from 39 in pure Col to 42 in the ColFn system, suggesting that Fn contributes to a denser, tighter network by accelerating fiber nucleation.

Thickness dynamics also varied with Col concentration and Fn incorporation. In pure Col at 2.88 mg/mL , the accumulation of thickness was relatively slow, with a half-maximum time ($t_{1/2,T}$) of 9.68 minutes and a final thickness of approximately $2.2 \mu\text{m}$. However, at a higher Col concentration (3.69 mg/mL), thickness formation accelerated markedly, as evidenced by a reduced $t_{1/2,T}$ of 4.59 minutes and a final thickness of about $2.8 \mu\text{m}$. The corresponding rate constants support these observations, with k_T increasing from 0.072 min^{-1} at 2.88 mg/mL to 0.151 min^{-1} at 3.69 mg/mL .

In the ColFn system at 2.88 mg/mL , thickness increased relatively quickly ($t_{1/2,T} = 6.5$ minutes); notably, while Fn speeds up the rate of thickness increase, the final thickness decreases to about $1.9 \mu\text{m}$. This suggests that Fn promotes a faster initial buildup of thickness, likely by enhancing early crosslinking or nucleation, but may also impose structural constraints that limit the ultimate scaffold thickness. The corresponding rate constants support these observations, with k_T increasing from 0.072 min^{-1} at 2.88 mg/mL to 0.107 min^{-1} for ColFn scaffolds.

Together, these results indicate that while a higher Col concentration is essential for efficient thickness buildup, Fn modulates the assembly process by accelerating early thickness development yet capping the final thickness.

Fiber length dynamics exhibited a pattern similar to the other parameters. At lower Col concentrations (both pure Col and ColFn at 2.88 mg/mL), the half-maximum times for fiber length ($t_{1/2,L}$) were comparable, around 6 minutes. However, the final fiber lengths differed markedly: at room temperature, the presence of Fn increased the final fiber length from approximately $30.7 \mu\text{m}$ in pure Col to about $53.3 \mu\text{m}$ in the ColFn system. This indicates that while Fn does not significantly change the elongation rate (as the exponential rate constants are similar at roughly 0.110 min^{-1}), it does enhance the ultimate fiber length.

In contrast, at the higher Col concentration of 3.69 mg/mL , fiber length reached half-maximum much faster ($t_{1/2,L} = 2.55$ minutes), and the exponential rate constant increased sharply to 0.272 min^{-1} , clearly demonstrating accelerated elongation. Interestingly, despite this acceleration, the final fiber length at 3.69 mg/mL (approximately $34.6 \mu\text{m}$) did not increase substantially compared to the 2.88 mg/mL condition, suggesting that higher Col concentrations accelerate early elongation kinetics but do not drastically alter the ultimate fiber length in the absence of Fn.

These combined results suggest distinct yet complementary roles for Col concentration and Fn during scaffold formation. Higher Col concentrations predominantly accelerate overall assembly by providing more molecular building blocks, which enhances the rate and extent of fiber formation and thickness development, evidenced by a decrease in the half-maximum time for fiber formation from 8.35 to 3.37 minutes, a more than two-fold increase in the rate constant (0.083 to 0.206 min^{-1}), and an increased fiber count (from 39 to 47).

In contrast, at 2.88 mg/mL , incorporating Fn leads to moderate but significant changes: it decreases the half-maximum time for fiber formation from 8.35 to 6.62 minutes and increases the rate constant from 0.083 to 0.105 min^{-1} , supporting our Chapter 3 findings that Fn accelerates fiber nucleation, and increases the fiber number from 39 to 42, indicating a denser, tighter network. Moreover, Fn accelerates the early buildup of scaffold thickness ($t_{1/2,T}$ drops from 9.68 to 6.50 minutes) but limits the final thickness (reducing it from approximately $2.2 \mu\text{m}$ to about $1.9 \mu\text{m}$), while also markedly enhancing final fiber length (from roughly $30.7 \mu\text{m}$ in pure Col to about $53.3 \mu\text{m}$).

At a higher Col concentration (3.69 mg/mL), early elongation is further accelerated ($t_{1/2,L} = 2.55$ minutes, $k_L = 0.272 \text{ min}^{-1}$), yet the final fiber length ($\sim 34.6 \mu\text{m}$) remains similar to that at 2.88 mg/mL in the absence of Fn. Therefore, while Col concentration primarily dictates the overall speed and scale of scaffold assembly, Fn fine-tunes the microstructural organization by enhancing fiber nucleation and increasing final fiber length, yet it also imposes structural constraints that limit maximum scaffold thickness.

5.5 Real-Time Imaging of ECM Organization Influenced by GFP-3T3 Fibroblasts

Following previous observations on fiber formation and scaffold structure at room temperature, the next phase of investigation focuses on the influence of cellular presence and activity at higher cell concentrations, specifically 10^6 and 10^7 cells/ml. In this chapter, Green Fluorescent Protein-expressing NIH/3T3 fibroblasts (GFP-3T3) were used to enable live-cell visualization during scaffold self-organization. To systematically explore cellular effects on ECM architecture, a controlled experimental setup was designed.

A custom mold with a removable dividing wall was employed, creating two distinct yet adjacent compartments: the left side contained a mixture of cells (10^6 cell/mL), Col (2.88 mg/mL), and Fn (0.2 mg/mL), while the right side contained an identical composition but without cells. Equal volumes were carefully injected into each compartment, ensuring a balanced surface to prevent solution flow. Immediately after injection, the wall was gently removed vertically to avoid disturbing the samples.

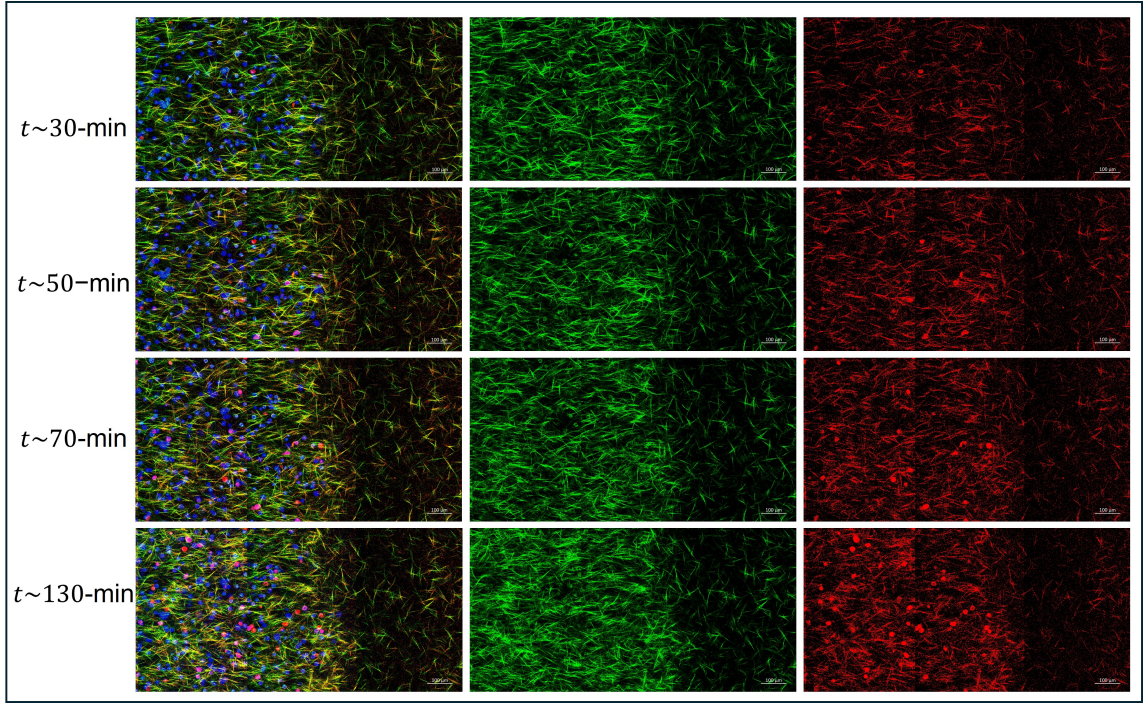


Figure 5.6: ECM evolution over time in presence of GFP-3T3 cells Time-lapse fluorescent micrographs of GFP-3T3-laden scaffolds at $t = 0, 30, 50, 70,$ and 130 minutes, illustrating progressive fiber formation and cell distribution. The left column shows all channels merged, the middle column displays collagen only, and the right column shows fibronectin only. Collagen (green), fibronectin (red), and cells (blue) are visualized through their GFP fluorescence. The overall field of view is $1.27\text{ mm} \times 0.638\text{ mm}$, and the scale bar represents $100\text{ }\mu\text{m}$.

Subsequent structural changes were captured through continuous z-stack and tile imaging. This methodology allowed real-time tracking of cell-driven alterations to Col and Fn distribution within the ECM, providing insights into how cells accumulate and reorganize ECM components under varying cellular concentrations. These results are illustrated in Figure 5.6. Each panel in Figure 5.6 shows a split scaffold in which the left half contains the GFP-3T3-laden collagen–fibronectin mixture (ColFnCell), and the right half contains a cell-free collagen–fibronectin region (ColFn). The original boundary between these halves was removed, allowing the two ECM domains to merge over time.

The presented results illustrate dynamic variations in ECM fiber density and organization, in-

fluenced by the presence or absence of GFP-3T3 cells, observed progressively from $t = 30$ min to $t = 130$ min. In regions populated by cells, ECM fibers display significant dynamic behaviour, including noticeable rearrangement and alignment over time. At a certain times, cells rapidly interact with both Col and Fn fibers, leading to the immediate or rapid formation of fiber clusters or islands.

Initially, Col fibers frequently form rounded clusters around the cells. However, these Col aggregations are often transient and gradually diminish or disappear over time, losing their distinct morphology. Not all Col clusters remain stable, some disperse completely, and in certain instances, Col clusters fail to form altogether, leaving only Fn islands. Fn islands, by contrast, form rapidly and are predominantly localized at or near cell positions. These structures often persist even after cells migrate away. Importantly, unlike Col clusters, Fn islands increase in number over time, suggesting they are significantly more stable and persistent within the ECM.

Conversely, in regions devoid of cells, ECM fibers remain relatively static, exhibiting limited morphological or spatial rearrangement. Both Col and Fn fibers maintain a uniform distribution, consistent with passive polymerization or crosslinking in the absence of cellular traction forces.

Overall, while ECM maturation occurs gradually in both cellular and cell-free regions, the presence of cells significantly enhances dynamic fiber reorganization. Cellular interactions actively influence local fiber density and alignment, generating structurally reorganized ECM regions that differ markedly from static, cell-free environments.

These observations suggest that cells might shift their interactions from Col to Fn as time progresses, potentially due to limited collagen-binding sites. This transition could support ongoing cell mobility and dynamic reorganization within the ECM. Additionally, fiber reorganization dynamics seem dependent on cell density; higher cell concentrations, which limit spatial freedom, result in more persistent Col aggregations resembling Fn islands. These observed ECM behaviours and their dependency on cell density and fiber interactions have prompted detailed investigations presented in the subsequent section.

5.5.1 Real-Time Analysis of Fn Island Formation: Initial Assembly, Maturation, and Fate

To further investigate how cells influence ECM organization, particularly the formation of islands or localized accumulations, we now shift our attention specifically toward the kinetic details observed through live imaging. Here, we concentrate on localized interactions occurring immediately upon mixing cells with collagen and fibronectin solutions, capturing the dynamic emergence of ECM islands from their earliest moments.

Col-only scaffolds containing cells were not imaged under the fluorescence conditions used here, as this experiment was specifically designed to visualize fibronectin organization and the formation of fibronectin-rich microislands. Because our focus in this chapter was on Fn-mediated reorganization rather than collagen-only island formation, equivalent Col-only images were not acquired for direct comparison.

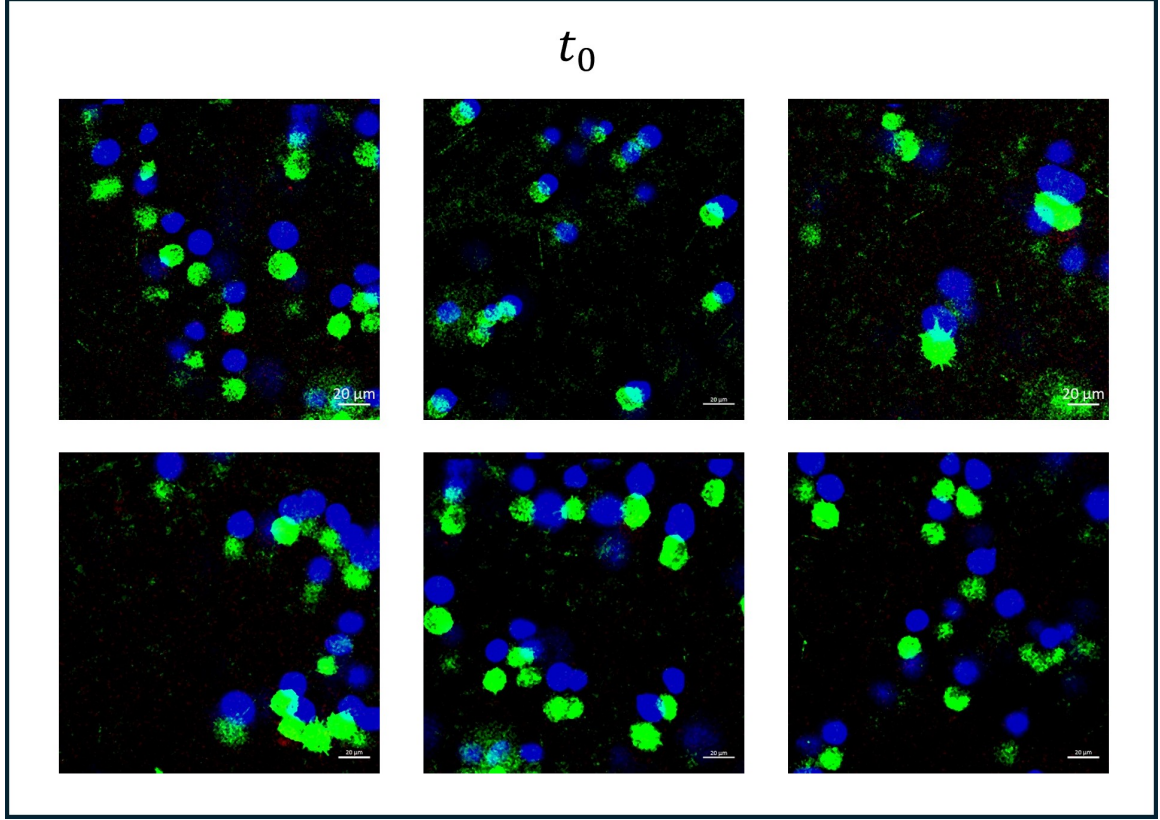


Figure 5.7: Confocal imaging of collagen and fibronectin fiber organization near individual cells. Representative fluorescent micrographs showing the evolution of ECM in the vicinity of individual GFP-3T3 cells at $t = 0$. Each panel corresponds to an initial time point from a different sample. The images combine multiple fluorescence channels: merged (collagen, fibronectin, and cells), collagen (green), fibronectin (red), and cells (blue) visualized through GFP fluorescence. At $t = 0$, collagen and fibronectin appear faint and largely unorganized, with minimal fiber formation visible. Some initial collagen aggregation can also be observed. Scale bars represent $20\ \mu\text{m}$.

Unlike experiments assessing structural outcomes after extended incubation periods, this study begins observing cell-ECM interactions at the initial time point ($t = 0$), prior to complete hydrogel formation (Figure 5.7). This early-stage observation provides crucial insights into initial cellular behaviours within a still-fluid matrix environment. Over time, distinct islands of Fn and Col form due to localized cell-driven rearrangements, which we aim to dissect with fine resolution and clarity. Rather than evaluating bulk matrix reorganization, this section specifically focuses on the detailed processes by which individual cells initiate and organize these ECM islands, elucidating the early spatial and temporal cues guiding ECM assembly.

Figures 5.7 and 5.8 provide clear visual evidence of the dynamic phases characterizing ECM organization by cells over short timescales. Immediately after cells, collagen, and fibronectin are combined and introduced under the microscope, ECM formation begins rapidly.

In the very initial moments, before the Col network is fully established, cells may experience mechanical stress due to the lack of stable attachment points. As a result, they rapidly adhere to the surrounding matrix and begin exerting traction forces on nearby fibrils [25]. These contractile forces lead to immediate, localized reorganization, with Col fibers becoming displaced and compacted in the pericellular region [25, 26]. Microscopically, this is observed as denser fiber clusters or aligned strands forming around the cell shortly after seeding. Such pericellular compaction is considered an inherent response to the mechanical imbalance between cell-generated tension and the matrix's elastic resistance [25, 27].

On compliant matrices, these clusters may form and relax dynamically in a “load-and-fail” man-

ner, as the weak matrix yields under cellular forces [26, 28, 29]. Acting as temporary anchors, these early Col aggregations help alleviate cellular stress by providing preliminary attachment points, allowing cells to begin organizing their microenvironment even in the absence of a pre-formed scaffold. However, due to their inherent mechanical instability, cells typically migrate away from these initial, weak interactions, continuously probing the matrix in search of more stable binding sites within the developing collagen network.

Following these initial mechanical events, cell motility and exploration play critical roles. If the initial Col islands fail to provide strong or appropriate binding sites for sustained adhesion and migration, cells will likely exert mechanical forces to detach themselves and move away in search of more suitable attachment points as the ECM continues forming. Moreover, active reorganization contributes to the disappearance of these islands, as cells remaining associated with these structures either reorganize the Col into longer fibrils or enzymatically break it down if the initial structure is suboptimal for creating a functional ECM.

This active reorganization is closely connected to mechanosensing processes mediated by integrin adhesions. In fibrous matrices, matrix deformability critically governs how cells perceive their environment; notably, soft collagen-like fiber networks allow cells to actively pull in and recruit fibers, thereby increasing the local density of ECM ligands (e.g., RGD sites) available to the cell [26, 30] (Figure 5.8). This fiber recruitment mechanism enhances focal adhesion assembly and integrin signaling, providing mechanical cues that sustain cell spreading, migration, and growth in three-dimensional environments. As reorganization progresses, cells continue reorganizing the collagen network and sensing mechanical feedback through integrin adhesions, further regulating their motility and survival [26].

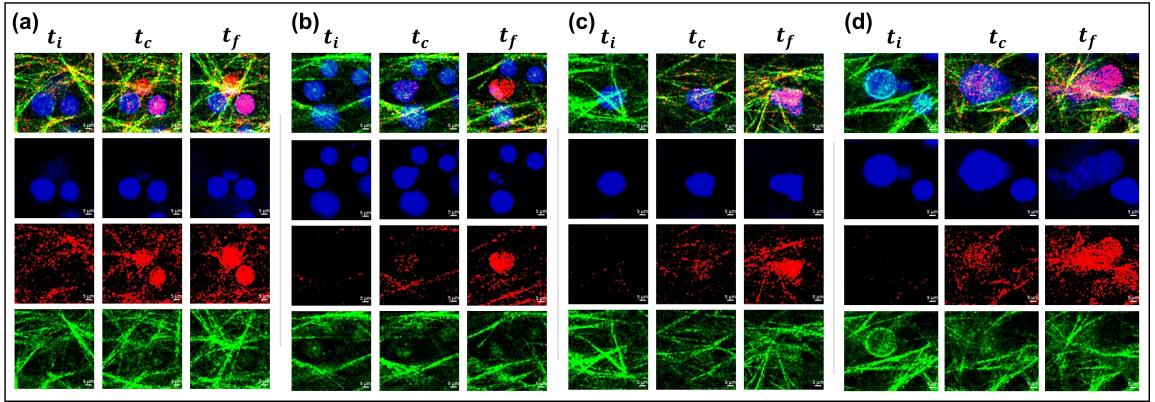


Figure 5.8: Time-lapse fluorescent images of GFP-3T3 fibroblasts in the ColFn ECM, high-lighting fibronectin island formation and collagen remodeling. (a)–(d) Each panel shows a different sample or field of view, with three time points: t_i (before fibronectin island formation), t_c (during fibronectin island formation), and t_f (after fibronectin island formation). Each row contains four images: a merged channel (top-left), a blue channel for cells visualized via GFP fluorescence, a red channel for Fn, and a green channel for Col. The time interval between t_c and t_f is about 3–5 minutes. In (a), a third cell drives Fn island formation and collagen fiber realignment; in (b), a migrating/attaching cell induces Fn island formation and Col reorganization; in (c), cell spreading triggers Fn island creation alongside Col remodeling; and in (d), a temporary Col island disappears, and the cell connects to a neighboring cell, resulting in Fn islands and further Col fiber bundling. Scale bars = 20 μm .

After the initial Col fiber network has formed, typically around 10–15 minutes as we discussed earlier, cells find themselves within an ECM that offers numerous opportunities for binding and movement. Beyond this point, however, they enter a more active and targeted phase of ECM reorganization. During this stage, cells begin to simultaneously deposit localized enrichments of both Col and Fn, forming distinct islands of these ECM components within the existing fibrous network [29].

While early interactions center around collagen reorganization, the formation of stable, Fn-rich

islands marks a shift toward a more sustained ECM reorganization strategy focused on long-term survival and multicellular coordination.

Cells actively create localized Fn-rich islands within the ECM primarily to enhance their resistance to anoikis, thereby ensuring survival in 3D environments [31]. Anoikis, a form of apoptosis triggered by insufficient cell-matrix attachment, is prevented by the deposition of Fn, which provides stable integrin-binding sites essential for cell survival. Through interactions with integrin receptors, particularly $\alpha_5\beta_1$, Fn activates critical signaling pathways such as focal adhesion kinase (FAK), providing essential biochemical survival cues [31].

Cells form these Fn-rich islands through a combination of localized secretion and active ECM reorganization. Initially, cells direct the secretion of Fn proteins into discrete regions around their immediate vicinity. Subsequently, they exert tractional forces on these ECM components via integrin receptors, physically pulling, aligning, and concentrating Fn fibrils into structured aggregates (Islands) [31]. Furthermore, these Fn fibrils link to Col fibers, creating a cohesive and mechanically stable ECM microenvironment.

This Fn-driven matrix reorganization facilitates stable cell-cell interactions by physically bridging neighboring cells, thus promoting the formation of multicellular clusters [29, 31]. These clusters further stabilize cell-cell junctions, increasing the expression of adhesion proteins such as E-cadherin and desmosomal components [31]. Without functional Fn or integrin $\alpha_5\beta_1$ interactions, cells fail to maintain stable clustering, underscoring Fn's pivotal role in cell viability and aggregate formation.

Continuous and coordinated deposition of Col and Fn enables precise, local-scale control over the ECM's composition and mechanical properties, creating specialized microenvironments that optimize cell function. Cells dynamically sense and respond to biochemical and mechanical cues within the ECM, and coordinated Col-Fn deposition supports efficient cellular adaptation. This dynamic process can generate signaling gradients, guide cell migration, and facilitate cellular communication and cooperation. Additionally, Fn-rich regions direct cell motility and ECM organization, influencing migration pathways and subsequent ECM reorganization, while surrounding collagen structures contribute to defining cell shape and stabilizing overall tissue architecture.

In this section, we examined the mechanisms underlying the formation of fibronectin and collagen islands, discussing both how and why cells actively create these structures. In the subsequent sections, we will explore the long-term effects of cellular activities on the ECM, particularly emphasizing ECM reorganization and cellular behaviours at high cell concentrations over extended periods, such as 24 hours.

5.5.2 Cell sedimentation

In 3D cell culture systems, cells are capable of exploring and migrating in all spatial dimensions. However, in practice, cells often exhibit preferential movement along the vertical (z) axis, predominantly driven by gravitational sedimentation [32] and cellular migration influenced by biochemical signals [33]. Figure 5.10 clearly illustrates this behaviour: two cells initially observed at a depth of 40 μm gradually descend to a 20 μm depth over approximately 110 minutes. During this interval, their lateral displacement in the x-y plane is negligible, indicating a clear dominance of gravitational forces in vertical displacement.

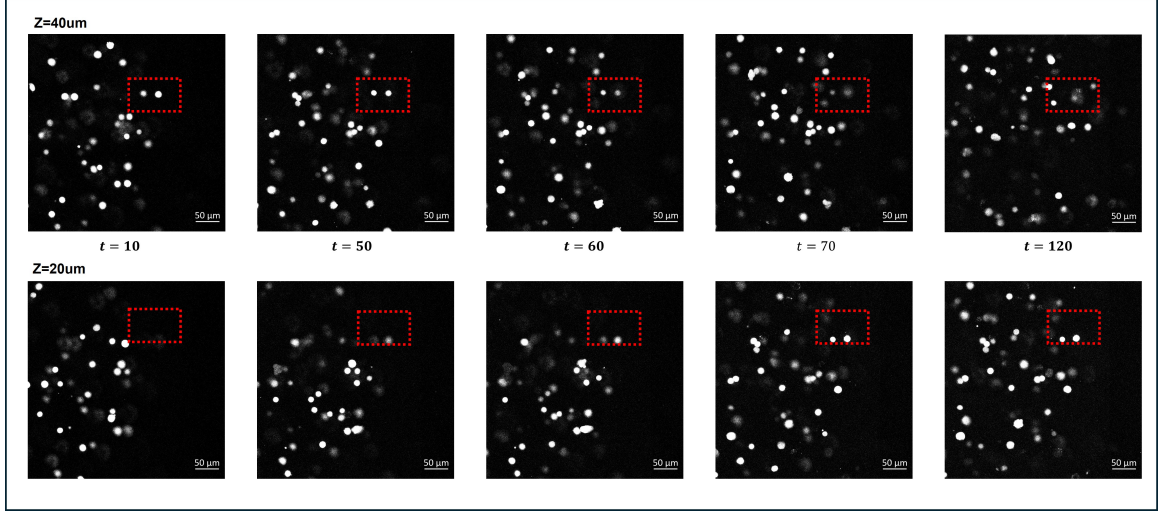


Figure 5.9: Time-lapse fluorescent micrographs illustrating predominantly vertical (z-axis) cell movement in a 3D environment. The top row ($z = 40 \mu\text{m}$) and bottom row ($z = 20 \mu\text{m}$) show the same field of view at multiple time points. The red dotted boxes highlight two tracked cells that, over approximately 110 minutes, migrate from the upper imaging plane to the lower one, suggesting that density effects drive a greater displacement in the z direction than in x - y . Scale bars = $50 \mu\text{m}$.

Several interconnected factors contribute to vertical cell sedimentation observed in the collagen-based hydrogel system in this study. One critical factor is the kinetics of hydrogel crosslinking and stiffness development. Although initial gelation occurred relatively quickly (within 10–15 minutes), the hydrogel achieves complete mechanical stiffness gradually, creating a semi-fluid environment in the early stages that allows gravitational forces to significantly influence cell movement [32].

Cell density notably influences sedimentation behaviour [32]. Higher cell densities ($10^6 >$ and 10^7 cells/mL) resulted in pronounced sedimentation compared to lower densities (10^5 and $< 10^6$ cells/mL). At elevated densities, increased cell-cell interactions and subsequent aggregation enhance collective settling behaviour, as cell aggregates have greater effective mass and diminished buoyancy. Although sedimentation was evident at all densities studied, the extent was significantly greater at higher concentrations as we will see in the following sections.

Interestingly, employing hydrogel inversion or flipping during gelation effectively reduced sedimentation in lower-density samples (10^5 cells/mL) but was less effective at higher cell densities (See Appendix). This observation emphasizes that alternative approaches, such as increasing hydrogel stiffness, adjusting the medium's viscosity, or using rotational bioreactors, may be required for uniform cell distribution at higher cell concentrations.

Recognizing the phenomenon of cell sedimentation is essential for experimental design and interpretation in 3D culture studies. Vertical cell settling affects the uniformity of cell distribution, potentially impacting cell-cell interactions, nutrient diffusion, ECM reorganization, and mechanical signaling within the hydrogel environment.

The experimental analysis presented in Figure 5.10 illustrates how cell counts evolve at distinct vertical positions ($z = 0, 20, \text{ and } 40 \mu\text{m}$) within a ECM over a 120 minute observation period. The curves represent changes in cell population numbers at each depth relative to their initial values, $N(t) - N(0)$.

To establish these results, a collagen–fibronectin solution was first injected into a mold placed on a microscope stage. After a brief period for initial gelation, cells were gently added on top. Despite careful pipetting intended to minimize mechanical disruption, rapid dispersion occurred due to droplet dynamics and density effects, causing cells to distribute quickly throughout the entire vertical range of the matrix. Cell positions at various depths were then quantified through imaging at defined intervals, revealing dynamic changes in population distributions across the layers.

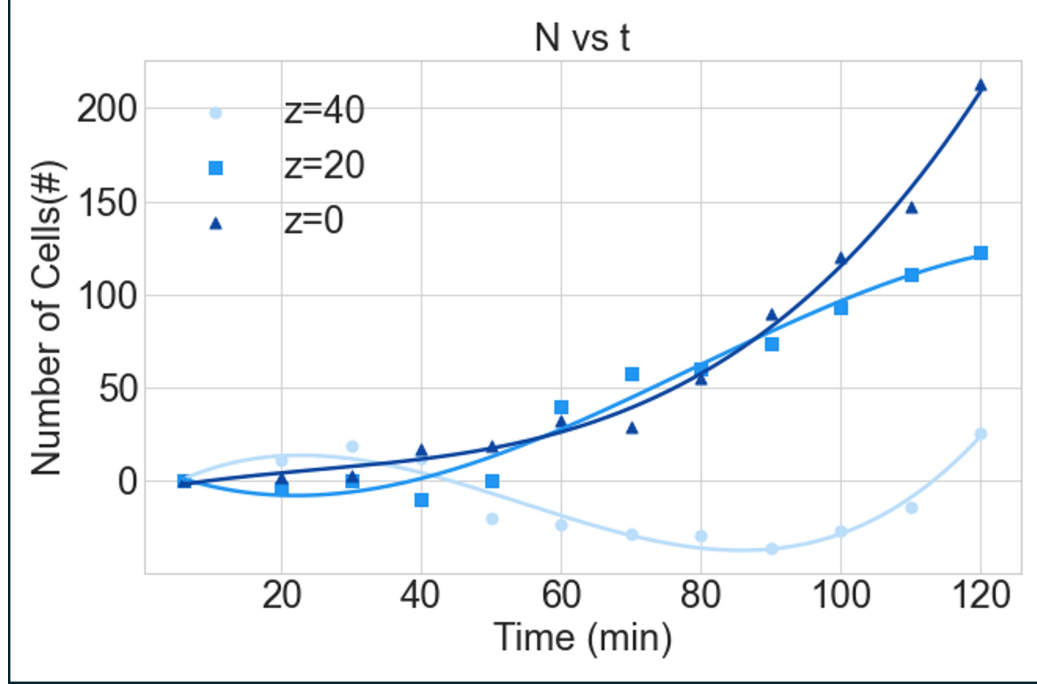


Figure 5.10: Time-dependent changes in cell counts at different z -layers ($z = 0, 20, 40 \mu\text{m}$) within a 3D collagen–fibronectin matrix. *Plotted values represent $N(t) - N(0)$ over 120 minutes, illustrating a pronounced increase in cell number at the bottom layer ($z = 0$), while higher layers exhibit slower growth or transient fluctuations. These data provide evidence of cell sedimentation and layer-specific structural influences on cell distribution.*

Analysis of the data reveals significant cell accumulation at the bottom layer ($z = 0 \mu\text{m}$), which displays the steepest increase in cell numbers throughout the observed timeframe. Because cells were introduced from above, gravitational forces combined with density gradients likely facilitated their downward sedimentation, resulting in a pronounced net increase at the lowest depth. lack of an exit point at the bottom also means cells that arrive there remain trapped, further contributing to cumulative cell numbers.

Conversely, the intermediate ($z = 20 \mu\text{m}$) and upper layers ($z = 40 \mu\text{m}$) show more gradual increases or even transient decreases before a secondary rise in cell counts. Such fluctuations suggest that cells traversing these layers may either continue downward under gravitational influence or encounter structural barriers within the hydrogel, such as dense collagen fibers or matrix regions with varying porosity. These structural elements can alter migration trajectories, causing cells to shift laterally, depending on local matrix conditions and cell–matrix interactions.

Overall, the observed trend strongly supports the conclusion that gravitational sedimentation dominates vertical cell distribution in this system. As we discussed, sedimentation typically becomes pronounced under conditions where the matrix polymerization is incomplete, or where its density and viscosity are relatively low, characteristics consistent with the collagen–fibronectin formulation used here [32, 34].

5.6 Conclusion

In this chapter, we explored how variations in Col concentration influenced both the kinetics and structural features of ECM assembly. Increasing Col concentration resulted in faster fiber nucleation and the formation of denser, more robust networks. This shift also enhanced fiber thickness and length, suggesting that Col availability plays a crucial role in defining the physical architecture of the matrix.

We also examined the effect of Fn on Col assembly. Adding Fn subtly accelerated the initial nucleation of Col fibers and increased the overall fiber number. However, Fn also appeared to limit fiber thickening while promoting greater fiber elongation, suggesting it fine-tunes the balance between lateral aggregation and longitudinal growth in the forming matrix.

Live-cell imaging provided deeper insights into the dynamic reorganization processes. We observed that early cellular traction led to rapid local compaction of collagen fibers, followed by the formation of mixed ColFn micro-islands. Over time, these structures matured into stable fibronectin-rich islands that persisted throughout the imaging period, indicating a sequence of pericellular reorganization events initiated by the cells.

Our data revealed that cells actively reorganized the ECM in an anisotropic manner, aligning Col fibers along force axes and stabilizing Fn-rich structures. These changes were not observed in cell-free regions, highlighting the importance of cellular activity in driving ECM organization.

Additionally, we observed a significant redistribution of cells within the 3D scaffold, driven by gravitational sedimentation. Over time, cells accumulated in the lower regions of the scaffold, with a corresponding depletion in upper layers. This phenomenon was especially pronounced at higher cell densities which is studied in the next chapter, and it emphasized that mechanical forces and matrix properties evolve concurrently during early ECM assembly, shaping both cell distribution and matrix structure.

These results establish clear links between macromolecular concentration, fiber formation kinetics, and emergent microstructures, while demonstrating that even within the first stages of assembly, cells can reorganize Col networks, nucleate Fn-rich islands, and redistribute vertically under gravity. The kinetic parameters observed here provide useful benchmarks for tuning gelation windows in cell-encapsulation protocols, and the live-imaging framework sets the stage for subsequent investigations of longer-term, cell-density-dependent reorganization and mechanics.

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Chapter 6

Exploring High Cell Concentration Effect on Collagen and Fibronectin ECM

6.1 Introduction

Fibroblasts play a critical role in reorganization the ECM during physiological and pathological processes, including development, wound healing, and fibrosis. In compliant, soft tissues (with stiffness typically ranging from 150–800 Pa), fibroblasts dynamically reorganize collagen and fibronectin (Fn) fibers, significantly influencing the ECM's structure and mechanical behavior [1]. Such remodeling is particularly pronounced during wound healing, where fibroblasts deposit a provisional, Fn-rich matrix and align collagen fibers, facilitating tissue contraction and defect closure [2, 3].

In 3D ColFn hydrogels, fibroblasts actively restructure the ECM depending on their density.

The ability of fibroblasts to remodel the ECM depends on both the matrix's mechanical properties and cellular context. Soft 3D collagen-based hydrogels (on the order of ~ 0.5 –2 kPa) permit robust cell-mediated remodeling, mimicking early-stage repair or healthy stroma, whereas stiffer matrices (10~kPa) resemble fibrotic or tumor microenvironments [4, 5]. Previous chapters indicated that fibroblasts at moderate densities maintain a predominantly fibrillar ECM structure with transiently formed Fn-rich islands. However, when fibroblast concentrations reach higher densities, we observed a marked structural shift. Fibroblast-generated mechanical forces trigger a phase transition from a continuous fibrous network to a clustered architecture characterized by persistent Col and Fn islands. Our findings reveal that this clustering phenomenon scales with cell density, becoming more prevalent as fibroblast concentration increases, and suggest a critical threshold at which matrix connectivity transitions from fibers to cellular clusters.

This chapter explores this high-density phenomenon in detail, emphasizing its significance and parallels with biological contexts such as wound healing and fibrosis. Notably, we observed fiber alignment around cavities or cell-free regions within the matrix, mirroring tissue repair processes. Fibers oriented radially toward these voids, resembling the "purse-string" contraction mechanism seen in wound closure, highlighting fibroblast-mediated mechanical guidance of ECM remodeling [6, 7].

Concurrently, fibroblast-driven reorganization substantially increased ECM stiffness. The collective traction forces from densely packed fibroblasts significantly enhanced local and global matrix rigidity, demonstrating that cellular forces substantially alter the initially soft hydrogel. These mechanical changes, accompanied by ECM structural rearrangements, have crucial implications for tissue engineering and disease modeling [1, 3, 8, 9, 10].

Finally, we recognize variations in power of reorganization behavior among different fibroblast subtypes (GFP and NIH-3T3 Cells). Future exploration into diverse cell populations could offer additional insights into how cell-specific factors modulate ECM organization and mechanics.

Collectively, this chapter provides a comprehensive examination of how high cell concentrations reshape ColFn ECM, with particular attention to the formation and implications of cellular clusters, fiber alignment, and matrix stiffening. These insights underscore the dynamic interplay between cell-generated mechanical forces, ECM architecture, and matrix stiffness, essential elements for understanding tissue reorganization in both physiological and pathological contexts.

6.2 Materials and Methods

In this study, a series of ColFn scaffolds were fabricated to evaluate their biological and mechanical characteristics (Figure 6.1). To evaluate mechanical properties, scaffolds were fabricated following a standardized protocol intended to replicate the conditions of live imaging experiments. Specifically, Col was uniformly mixed with Fn and cells, then allowed to polymerize at a controlled room temperature of approximately 21°C for 15 minutes to match the structural conditions observed microscopically. Subsequently, samples were transferred into an incubator at physiological conditions (37°C) for further polymerization and structural stabilization for 24 hours to achieve complete polymerization and optimal mechanical stability.

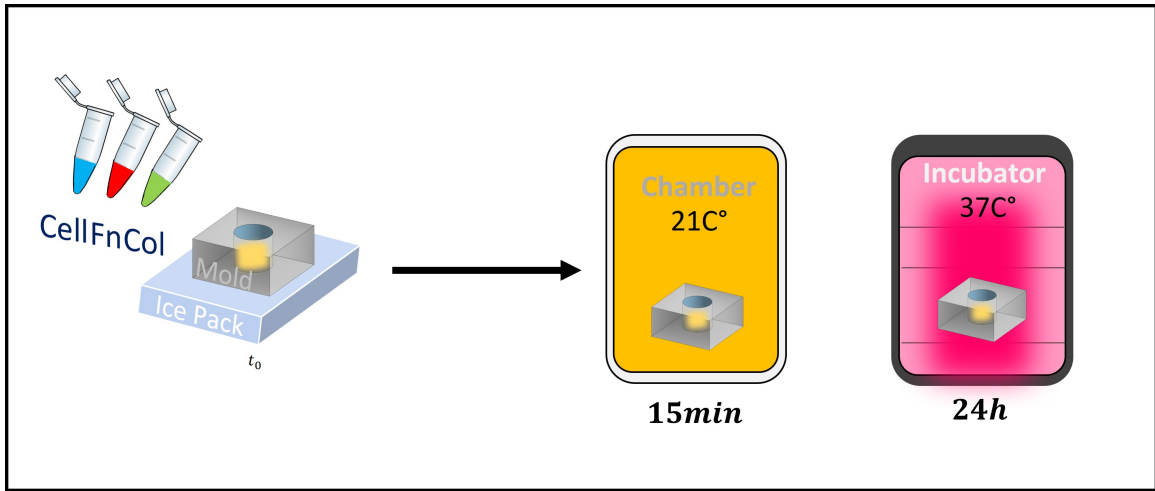


Figure 6.1: Scaffold fabrication method Schematic representation of scaffold preparation for live imaging and mechanical analysis. Collagen, fibronectin, and cells were mixed under sterile conditions on an ice pack. The scaffold mixture (collagen, fibronectin, and cells) was initially prepared on ice, then polymerized at 21°C for 15 minutes followed by incubation at 37°C for 24 hours for mechanical testing.

The following sections provide detailed analyses of each scaffold type, including specific fabrication parameters, structural characteristics, and mechanical property assessments.

6.2.1 Cell Culture Experiments

As in previous experiments, cell-containing samples were prepared by mixing cells thoroughly with the Col solution before adding the mixture into molds. Samples were first incubated for 1 hour. After this initial incubation, samples were flipped for an additional 2 hours, a critical step based on principles discussed in previous chapters, with intention to have a uniform structural integrity and promote even cell distribution. To enhance the clarity of observations and validate results, two distinct cell populations were utilized: GFP-expressing NIH 3T3 cells, selected for their robust GFP fluorescence, and regular NIH 3T3 cells, stained with Alexa Fluor Plus 488 Phalloidin.

Briefly, after cell culture and scaffold formation, samples were fixed using a 4% paraformaldehyde (PFA) solution in PBS for 10–15 minutes at room temperature. Samples were then gently washed three times with phosphate-buffered saline (PBS) to remove residual fixative. Next, samples were permeabilized by adding PBS containing 0.1% Triton X-100 for 10–15 minutes at room temperature,

allowing the stain to effectively penetrate the cells. The staining solution was prepared by diluting Alexa Fluor Plus 488 Phalloidin according to the manufacturer’s recommendations (1:1000 in PBS). Between 70–150 μL of the staining solution (depending on the sample size) was applied to fully cover each sample. Samples were then incubated for 20 minutes at room temperature in the dark to prevent photobleaching. Finally, samples were rinsed three times with PBS for 5 minutes each to remove any unbound phalloidin before imaging.

6.2.2 Structural Characterization of Collagen Scaffold Samples Imaging

Imaging of Col, Col-Fn, and Col-Fn cell-laden scaffolds was quantified using a laser scanning fluorescence confocal microscope (Zeiss LSM880, Zeiss, Munich, Germany) with a $20\times$ objective. For imaging, at least ten randomly selected regions of each sample were scanned in three dimensions ($1.27\text{ mm} \times 1.27\text{ mm} \times 0.05\text{ mm}^3$, with an $8\text{ }\mu\text{m}$ gap in the z-direction) and analyzed using Zen Blue software. In this thesis, all structural measurements and analyses presented are performed on Col fibers. This approach was chosen because all scaffold samples investigated here are Col-based, and the structural organization of Fn was observed to closely follow and conform to the underlying Col fiber architecture.

Due to the duration of the experiment and to prevent fiber degradation, samples were fixed by immersing them in phosphate-buffered saline (PBS) (*Life Technologies*) and formalin (*Sigma Aldrich*) for 20 minutes before imaging.

6.2.3 Mechanical Characterization of Scaffolds by Rheometry

The mechanical properties of the scaffolds were assessed using a parallel-plate rheometer (MCR301, Anton Paar) equipped with 12 mm diameter circular plates, matching the scaffold dimensions. Before testing, each sample was fully hydrated. To ensure accurate measurements and prevent slippage between the sample and rheometer plates, samples were glued to both plates using Permabond Instant Adhesive 102, a general-purpose adhesive with medium viscosity. Additionally, the rheometer plates were sandblasted to improve adhesion further.

Samples were individually transferred from the incubator directly to the rheometer to minimize changes in hydration and temperature. Experiments were conducted at room temperature ($23\text{--}24^\circ\text{C}$), and deionized water was regularly added around each sample’s perimeter during testing to maintain hydration. Samples were subjected to small-amplitude oscillatory shear tests to evaluate viscoelastic properties, specifically measuring the storage modulus (G') and loss modulus (G'').

The detailed experimental procedures followed standard protocols as described in Chapter 3. For additional experimental details, please refer to Chapter 3.

6.2.4 Statistical Analysis

Statistical analyses were performed using one-way ANOVA with Tukey’s post hoc test and Student’s t-test to determine statistical significance between conditions, using GraphPad Prism. In all cases, $p < 0.05$ is indicated by a single star (*), $p < 0.01$ by a double star (**), and $p < 0.001$ by three stars (***).

6.3 Fn Island Formation by Both GFP and NIH Cells

To further investigate how cells influence ECM organization, particularly the formation of islands or localized accumulations, we prepared scaffolds using varying cell concentrations and two different cell types. By systematically altering cell density, we aim to determine whether higher cell numbers consistently enhance or alter these island-like structures, and to see whether different cell lines exhibit similar reorganization behaviors. The following results illustrate these effects, highlighting how both cell type and concentration contribute to local ECM rearrangements.

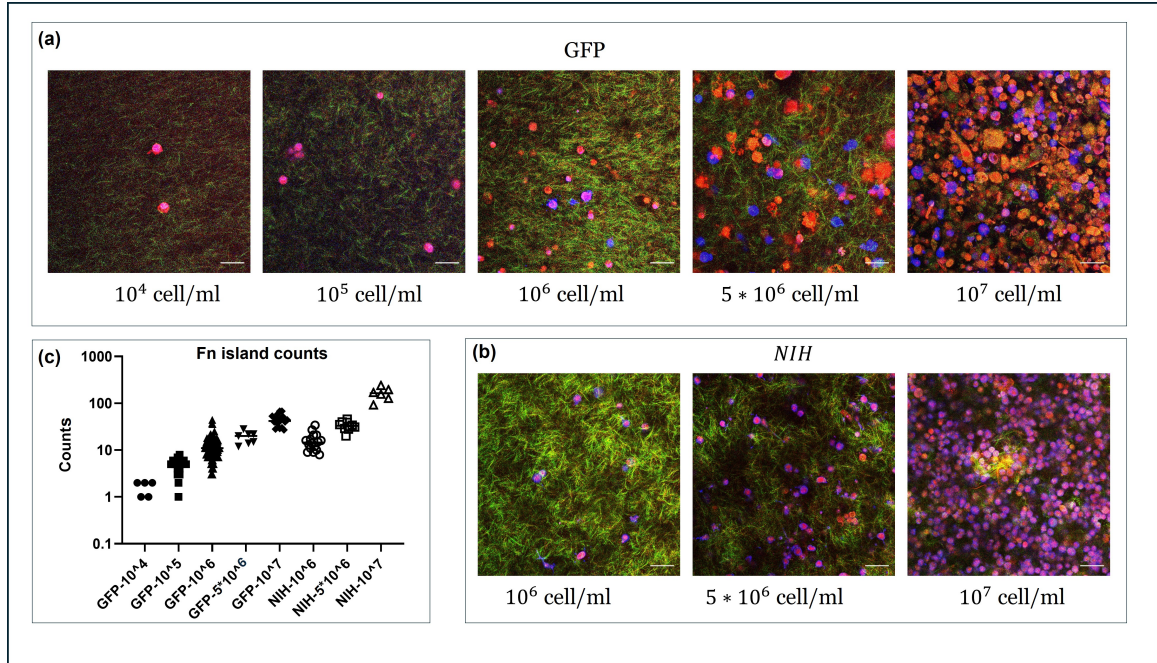


Figure 6.2: Fibronectin island formation at varying cell concentrations and cell types (a) Representative fluorescent micrographs of scaffolds seeded with GFP-labeled cells at increasing cell densities (from 10^4 to 10^7 cells/ml). Fibronectin “islands” (red) become more frequent and prominent as cell density increases. Collagen is shown in green, and cell nuclei are shown in blue. Composite colors, such as purple, appear where cells overlap with fibronectin or collagen. (b) Similar images for NIH 3T3 cells at 10^6 and 3×10^6 cells/ml, and 10^7 cells/ml showing a comparable trend in fibronectin clustering. (c) Quantification of fibronectin island counts across different cell densities and cell types confirms that higher cell numbers generally lead to increased fibronectin island formation; The scale bar represents $50 \mu\text{m}$.

Figure 6.2 illustrates how both GFP-labeled cells and NIH cells influence Fn and Col islands across a range of cell concentrations. The samples shown in the figure were incubated for 24 hours and subsequently fixed prior to imaging. This phrasing smoothly connects your description of the figure with the new experimental details.. In panel (a), at lower cell densities, most Fn islands appear at the exact locations where cells reside. As the cell concentration increases, some new islands form at sites initially unoccupied by cells suggesting that cells have migrated and reorganized the ECM elsewhere. Notably, at the highest cell density (10^7 cells/mL), the ECM structure is heavily disrupted by the large number of cells, resulting in a predominance of island-like clusters rather than continuous fibers.

Although GFP and NIH cells display similar trends, GFP cells, being larger and more aggressive, produce a more pronounced ECM disruption. Consequently, NIH cells, which exert a less disruptive influence, were selected for subsequent experiments to achieve more consistent and controlled outcomes. A histogram presented in panel (c) quantifies island counts across a $420 \mu\text{m} \times 420 \mu\text{m}$ field of view, confirming that higher cell densities correlate directly with increased Fn and Col island numbers, reinforcing the idea that elevated cellular concentrations amplify ECM reorganization.

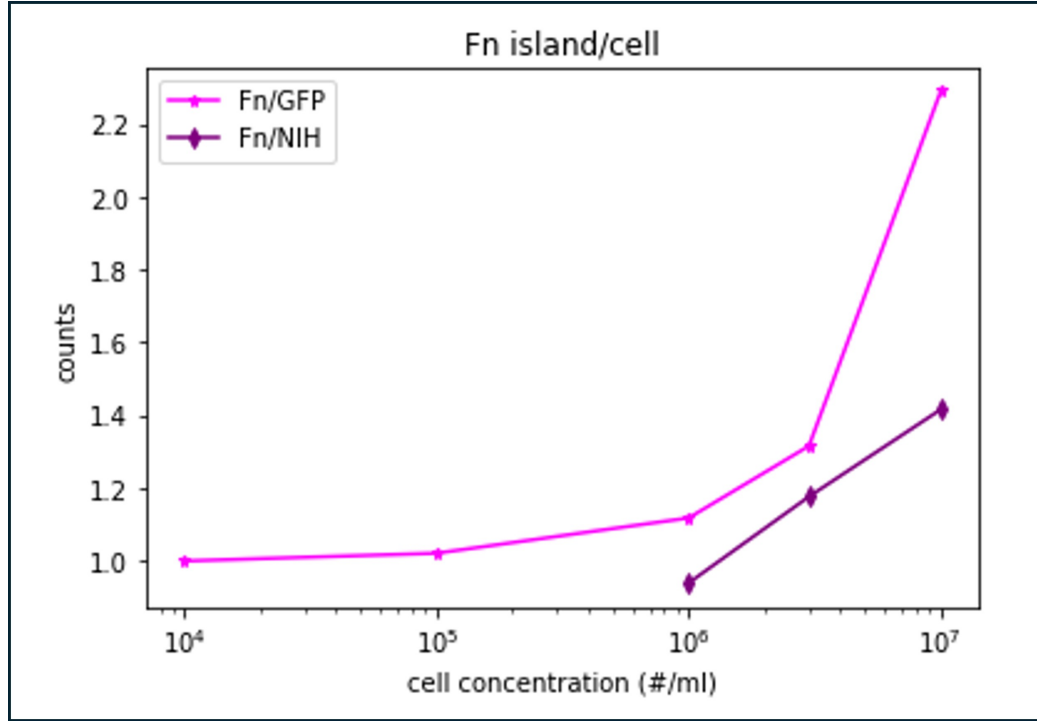


Figure 6.3: Fibronectin island formation per cell at increasing cell concentrations. *Quantification of fibronectin island counts per cell across a range of cell concentrations (10^4 to 10^7 cells/ml) for GFP-labeled cells (pink) and NIH 3T3 cells (purple). Both cell types show an increase in fibronectin islands per cell with rising density, indicating heightened ECM remodeling activity. The effect is more pronounced in GFP cells, suggesting more aggressive remodeling behavior compared to NIH cells.*

Complementing Figure 6.2, the data in Figure 6.3 further quantifies Fn island formation, showing that the number of Fn islands per cell rises with increasing cell concentrations. This phenomenon is particularly pronounced for GFP-labeled cells, aligning with earlier observations of their aggressive reorganization behavior. Nonetheless, NIH cells also demonstrate an upward trend in island formation at elevated cell densities, underscoring that both cell type and cell density significantly influence the ECM reorganization process.

6.4 Impact of Cell Concentration on ECM Reorganization and Spatial Organization

Building upon the previous chapter, which examined real-time ECM polymerization dynamics during early gel formation, this section instead focuses on the long-term structural outcomes of cell-mediated ECM reorganization. Here, we investigate how varying cell concentrations influence ECM organization by preparing samples with initial densities ranging from 10^6 to 10^7 cells/mL, incubating them for 24 hours, chemically fixing them, and subsequently imaging the resulting ECM architecture.

Understanding how cells collectively reorganize the ECM as their density increases is essential. At low cell densities, Cells in Col engage in matrix interactions via dendritic processes, extending long protrusions to probe and connect without globally reorganizing the matrix [11]. However, as cell concentration surpasses a critical threshold, the synchronized traction forces generated by densely packed cells overcome the matrix's mechanical resistance, leading to localized compaction and fiber clustering. Notably, at higher concentrations approaching 10^7 cells/mL, cell-driven reorganization induces the formation of phase-separated regions, significantly altering the ECM's microarchitecture. These observations underscore the importance of understanding how cell concentration affects ECM behavior and stability.

Additionally, this section explores differences between the top and bottom regions of the samples, where cell sedimentation and density gradients lead to distinct structural outcomes. The impact of higher cell concentrations on ECM stiffness will also be addressed later in the results to emphasize the mechanical implications of these observations. Understanding these mechanical changes is essential, as they can influence cellular behaviors such as migration and invasion, which are critical in both normal physiological processes and disease states

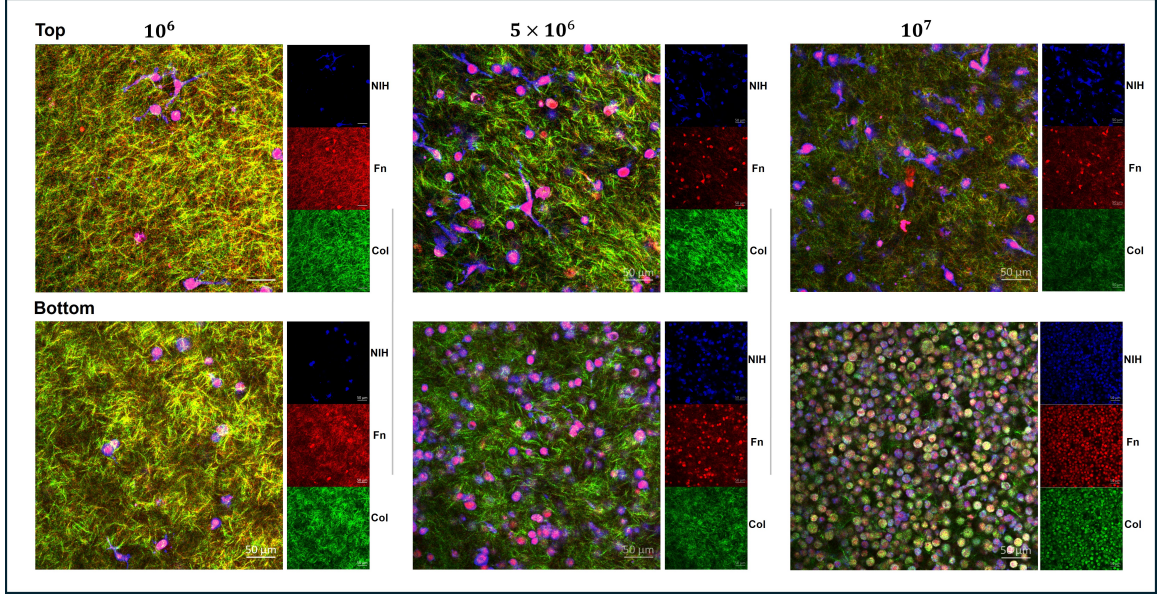


Figure 6.4: Fluorescence Microscopy Analysis of ECM Remodeling at Different Cell Concentrations. *Fluorescence microscopy images of NIH 3T3 cells (blue), fibronectin (red), and collagen (green) at the top (top row) and bottom (bottom row) surfaces of samples seeded with 10^6 (left), 5×10^6 (center), or 10^7 cells/sample. The smaller panels on the right of each image show the individual fluorescent channels. Higher cell concentrations accentuate sedimentation effects and ECM remodeling, with increased cell density and structural heterogeneity at the bottom surfaces. Scale bars = $50 \mu\text{m}$.*

Figure 6.4 illustrates fluorescence microscopy images of ColFn samples at different initial cell concentrations (10^6 , 5×10^6 , and 10^7 cells/ml). Images from both the top and bottom layers of each sample reveal differences in cellular distribution and ECM organization.

Due to sedimentation effects previously discussed, cells exhibit a greater density at the bottom surfaces compared to the top surfaces. This trend becomes increasingly pronounced at higher cell concentrations, leading to structural heterogeneity within the scaffold. At the top layers, where cells have more available space, they exhibit extensive spreading and less constrained alignment.

In contrast, cells at the bottom layers, particularly at higher concentrations (5×10^6 and 10^7 cells/sample), display less alignment and demonstrate significant reorganization of the ECM. This reorganization, driven by cellular traction forces, results in the directional orientation and organization of collagen fibers.

It should be noted that the apparent fiber alignment observed within the high cell-density regions of the scaffolds was assessed qualitatively from fluorescence confocal images. No quantitative orientation analysis (e.g., structure tensor analysis) or birefringence-based measurements (e.g., crossed-polarized light microscopy or DIC) were performed, as these imaging modalities were not part of the experimental workflow. Therefore, the alignment described here reflects visual structural trends rather than measured orientation indices. While polarization-based imaging could, in principle, provide additional confirmation of collagen alignment within these dense regions, this remains an opportunity for future work.

At the highest concentration (10^7 cells/ml), Fn and Col clustering become dominant, transforming the ECM from a predominantly fibrous network into distinct clustered islands. This transition

is driven by a positive feedback mechanism, wherein increased cell density amplifies cellular contractility and Fn secretion, which in turn stabilizes and expands clustered regions [7]. Even at lower densities, fibroblasts can nucleate localized Fn-Col aggregates, but as cell numbers rise, these clusters grow in size and prominence, ultimately becoming the dominant structural feature of the matrix. These islands represent a new structural phase characterized by localized aggregations rather than uniformly distributed fibers. This behavior echoes the concept of a percolation threshold or jamming transition in fibrous matrices, where cell-generated forces drive the system from a disperse, interconnected network into a clustered state [7]. Fibronectin integration is essential for stabilizing these clusters, as evidenced by studies where disruption of Fn assembly prevented island formation [7].

Moreover, increased cell concentration contributes directly to greater ECM heterogeneity, characterized by noticeable cavities or depressions within the scaffold. These cavities, identifiable as darker regions in the collagen network, arise due to both cellular clustering and active reorganization processes, further highlighting the complex interplay between cell density and ECM structural dynamics.

Interestingly, Col fibers around these voids (Figure 6.8 and Figure A9) aligned radially inward, resembling cell-driven centripetal alignment during wound healing [2, 6]. Cells at the cavity interface likely experience reduced mechanical resistance toward the void, promoting inward pulling and fiber organization akin to 'purse-string' contraction [6, 7].

The observed transformation from a predominantly fibrous ECM to distinct clustered islands at the highest cell concentration indicates a phase transition within the ECM architecture. This structural shift suggests that once a critical cell density threshold is reached, the ECM undergoes qualitative changes that have profound implications for tissue functionality and pathology.

Also, this induced heterogeneity in ECM structure, characterized by clustering and cavity formation, significantly influences cell behavior by altering local biomechanical properties and biochemical signals. Such alterations can impact cell signaling pathways, migration patterns, and differentiation processes, thereby influencing overall tissue development and function [12, 13, 14].

Therefore, understanding these structural dynamics is particularly crucial for tissue engineering applications, where uniform ECM properties are typically desired to ensure consistent cell behavior and tissue outcomes. Furthermore, similar ECM reorganization processes are also relevant in pathological contexts, such as fibrosis and cancer, where changes in ECM architecture can profoundly affect disease progression and therapeutic responses [13].

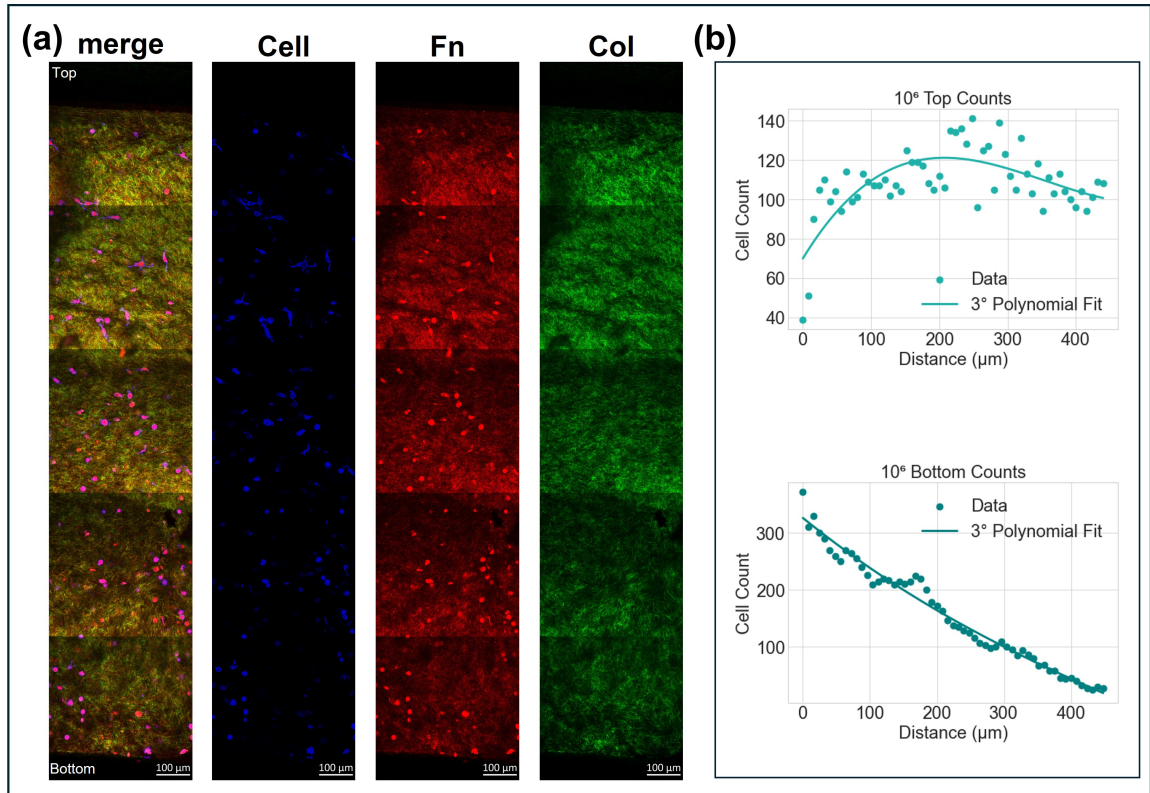


Figure 6.5: Cross-sectional analysis of a 3D scaffold seeded with 1×10^6 cells/ml. (a) Fluorescence images of a slice rotated to reveal the z - x plane, showing merged signals (merge) and individual channels for cells (blue), fibronectin (red), and collagen (green) from the top (left) to bottom (right) regions. Although partial compression occurred during sectioning, the distribution and ECM organization are still visible. (b) Quantitative cell counts obtained by analyzing x - y images ($850 \mu\text{m} \times 850 \mu\text{m}$ area) at multiple z -levels. The plotted data illustrate how cell density varies from top to bottom, indicating a gradient influenced by sedimentation and ECM remodeling. Scale bars = $100 \mu\text{m}$.

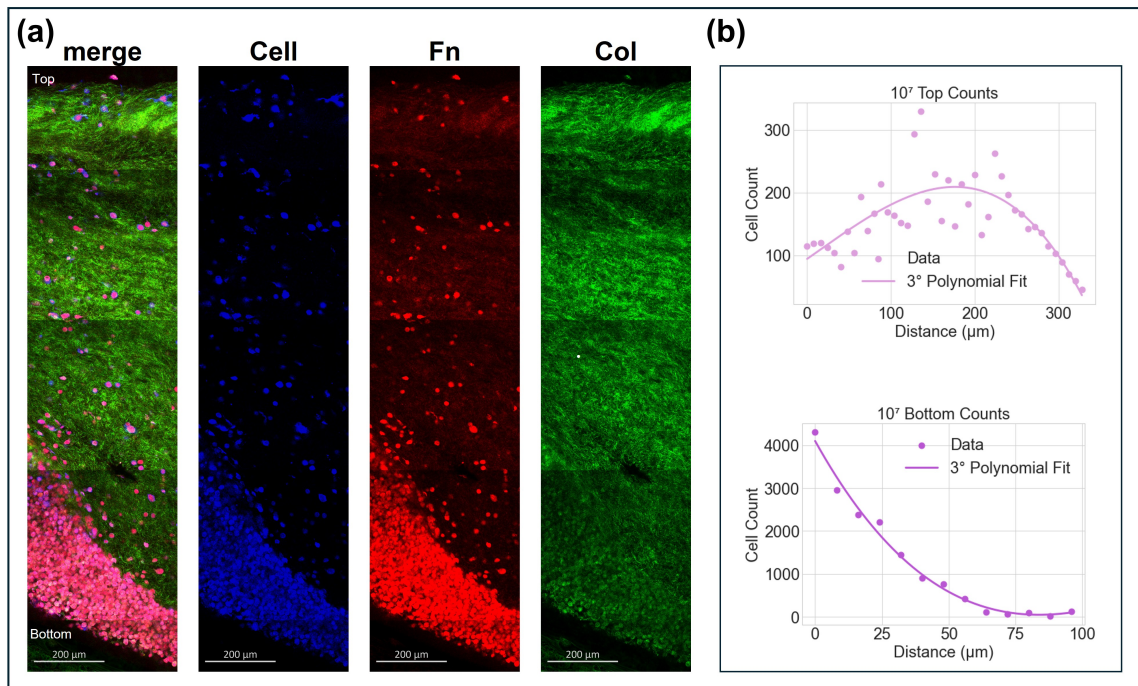


Figure 6.6: Cross-sectional analysis of a 3D scaffold seeded with 10^7 cells/ml. (a) Fluorescence images of a slice rotated to show the z-x plane, with merged (merge) and separate channels for cells (blue), fibronectin (red), and collagen (green). A higher initial cell concentration leads to more pronounced clustering and ECM remodeling, especially near the bottom. (b) Cell count distribution plotted, similarly derived from thresholded x-y images ($850\ \mu\text{m} \times 850\ \mu\text{m}$ area) at various z-positions. Compared to the 10^6 condition, the 10^7 sample exhibits a steeper gradient, underscoring the impact of higher seeding density on cellular organization through the scaffold thickness. Scale bars = $100\ \mu\text{m}$.

To visualize how cells and ECM components distribute throughout the sample thickness, we conducted cross-sectional imaging combined with quantitative analysis at multiple depths along the z-direction (Figures 6.5 and Figure 6.6, panels a and b). After initially capturing the x-y plane views of the 3D ECM structures, samples were sliced, and each slice was rotated to image the z-x plane under fluorescence microscopy. This method effectively provided a side view of the scaffold, revealing the extent of cellular and ECM infiltration through its thickness. It should be noted that some structural compression unavoidably occurred during slicing, potentially affecting fiber alignment and pore size; however, the primary distribution characteristics of cells and ECM remained clearly observable.

Cross-sectional observations consistently indicated greater cell accumulation near the scaffold's bottom surface at higher cell seeding concentrations, highlighting sedimentation effects occurring before complete matrix polymerization. This sedimentation-driven accumulation corresponds well with the 2D observations presented in Figure 6.4 and can enhance local remodeling forces exerted by cells.

To complement qualitative observations from cross-sectional imaging, systematic cell density quantification was performed on x-y plane images obtained at various depths (z-levels). Using FIJI's particle analysis, cell nuclei were thresholded and counted within an $850\ \mu\text{m} \times 850\ \mu\text{m}$ field of view for each depth. Plotting these counts against the sample depth generated detailed cell-density distribution profiles. At lower cell concentrations (10^6 cells per sample), distribution curves exhibited gradual changes, reflecting relatively even infiltration but a lower overall density. Conversely, higher cell concentrations (10^7 cells per sample) displayed sharper peaks, predominantly near the bottom, indicating preferential cell sedimentation or proliferation in these regions.

Moreover, the collagen network exhibited cavities or cracks (Figure 6.6-panel a), signaling a structural transition from a predominantly fibrous architecture to distinct clustered islands. These

observations strongly suggest a phase transition within the ECM structure, prompting deeper exploration into the mechanisms and consequences of high cell-density effects on ECM architecture.

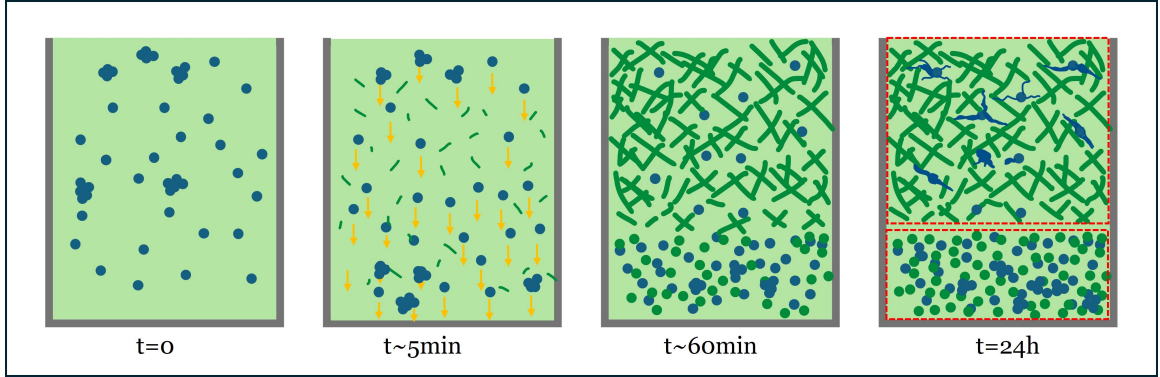


Figure 6.7: ECM Phase Transition Mechanism at High Cell Density. *The schematic illustrates how a ColFn scaffold evolves over time under high cell density. Light-green shows the initial collagen solution, blue denotes cells, green represents Col/Fn fibers or island structures, and orange arrows indicate the combined effects of gravity and cell signaling forces. Initially, cells are dispersed in a soft matrix; as they sediment and remodel the ECM, dense cell clusters form and stiffen the local environment, eventually leading to cracks or cavities that signal a phase transition in the matrix structure.*

Figure 6.7 illustrates the proposed mechanism whereby high cell density, influenced by gravitational sedimentation and cell-mediated ECM reorganization, induces this phase transition. Initially, cells uniformly dispersed within the unpolymerized ColFn solution settle toward the bottom due to gravity, increasing local cell density in these regions. Enhanced cell-cell interactions at higher densities stimulate fibronectin secretion and collagen fiber reorganization, forming compact, stiff microdomains, or islands, interspersed by softer, fibrous areas with fewer cells, allowing more cellular spreading [15].

Over extended incubation periods (e.g., 24 hours), significant mechanical reorganization of the ECM is driven by high local cell density at the bottom. Continuous cellular forces, arising from gravity and intracellular signaling, create substantial stresses within the matrix [15]. When these stresses surpass the mechanical limits of the surrounding softer fibrous network, structural breakdown occurs, forming visible cavities or cracks. This ECM reorganization and structural failure mark a critical phase transition from a continuous, fibrous network to a heterogeneous structure composed of stiff, cell-rich islands separated by voids.

Our hypothesis that high cell density drives ECM reorganization into a phase transition, characterized by stiff islands interspersed with cavities, parallels feed-forward mechanistic loops seen in pathological conditions such as fibrosis and cancer [15]. In these diseases, enhanced ECM production creates stiffer microenvironments, which further amplify cellular responses and ECM deposition, perpetuating a detrimental cycle. Similarly, in our high-density samples, sedimented cells initiate localized ECM stiffening and structural failure, mirroring fibrotic tissues and tumor desmoplasia. Recognizing these parallels underscores the validity of our hypothesis and suggests potential therapeutic strategies by targeting mechanotransduction pathways or proteolytic reorganization processes to disrupt these self-reinforcing cycles [15, 16].

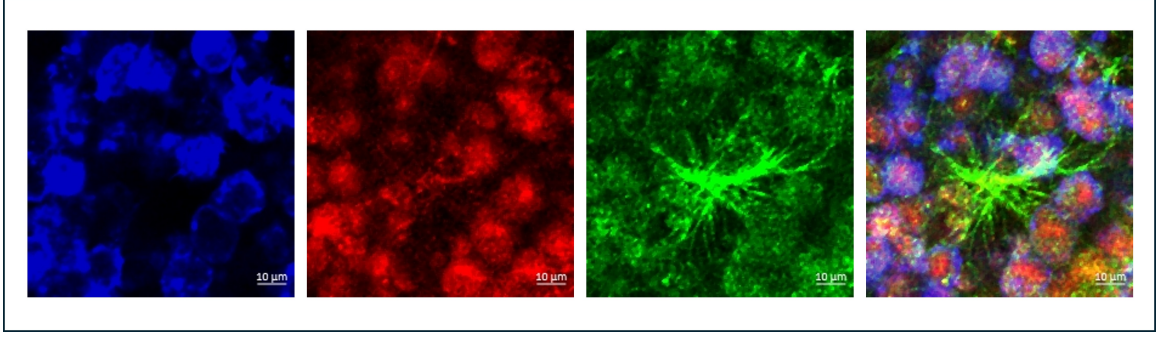


Figure 6.8: Active ECM Reorganization and Void Closure in Dense Cellular Microenvironments. *High-resolution fluorescence image showing a localized cavity within a densely populated cell region. Cells at the cavity edges (blue) are seen directing the alignment of collagen fibers (green) radially into the gap, while fibronectin (red) appears as discrete island-like clusters. This pattern, reminiscent of wound-healing processes, suggests that cells are actively reorganizing the ECM to mechanically bridge the gap and restore tissue continuity. Scale bars = 10 μm .*

Interestingly, the observed phase transition and subsequent cavity formation in our system closely resemble aspects of wound healing (Figure 6.8), particularly the proliferative phase. During wound healing, fibroblasts migrate into damaged areas and deposit ECM proteins, primarily collagen, forming the structural backbone of granulation tissue. These cells actively generate mechanical forces, aligning collagen fibers to bridge gaps and facilitate wound contraction, restoring structural integrity [17, 18]. Similarly, in our high-density samples, cells near cavity edges align collagen fibers radially toward the voids, attempting to mechanically stabilize and close the gaps, mimicking fibroblast behavior during wound contraction. Additional figures at larger scales illustrating this wound healing-like behavior are provided in the appendix. Notably, fibronectin, while abundant, primarily forms clustered islands without displaying the same directed alignment as collagen, highlighting collagen’s pivotal role in providing tensile strength for gap closure. This parallel underscores the broader relevance of our findings, connecting cellular dynamics and ECM reorganization observed in our experimental system to fundamental biological processes and potential pathological scenarios like fibrosis [15].

6.5 Rheological Characterization of High Density ECM Samples

Building on our earlier findings on ECM reorganization under different cell densities, we now turn our focus to the rheological properties of the samples seeded at 1×10^6 and 1×10^7 cells. In the previous section, we demonstrated that high cell density promotes a phase transition within the ECM, leading to the formation of stiff, island-like regions interspersed with cavities. These structural changes, driven by cellular sedimentation and active reorganization, are expected to significantly impact the mechanical behavior of the ECM. This cell-driven stiffening of the ECM has been well documented, where fibroblast traction forces nonlinearly stress-stiffen fibrillar matrices like collagen and fibrin [8, 9]. Locally, fibroblast-induced tension aligns and stiffens the surrounding fibers, a process that accumulates with increasing cell density to produce bulk mechanical reinforcement. This transition from a soft to a stiffer, more anisotropic ECM is a hallmark of high-density cellular environments [8].

In this section, we aim to quantify these effects by measuring the viscoelastic properties of the samples using rheology. Our analysis will compare the stiffness, elasticity, and overall mechanical response of the high-densities ECM constructs, highlighting how variations in cellular organization and ECM structure correspond to changes in mechanical performance. The insights obtained here will help us understand the interplay between cellular dynamics and ECM biomechanics, providing a foundation for linking our observations to potential pathological processes such as fibrosis and

tumor progression.

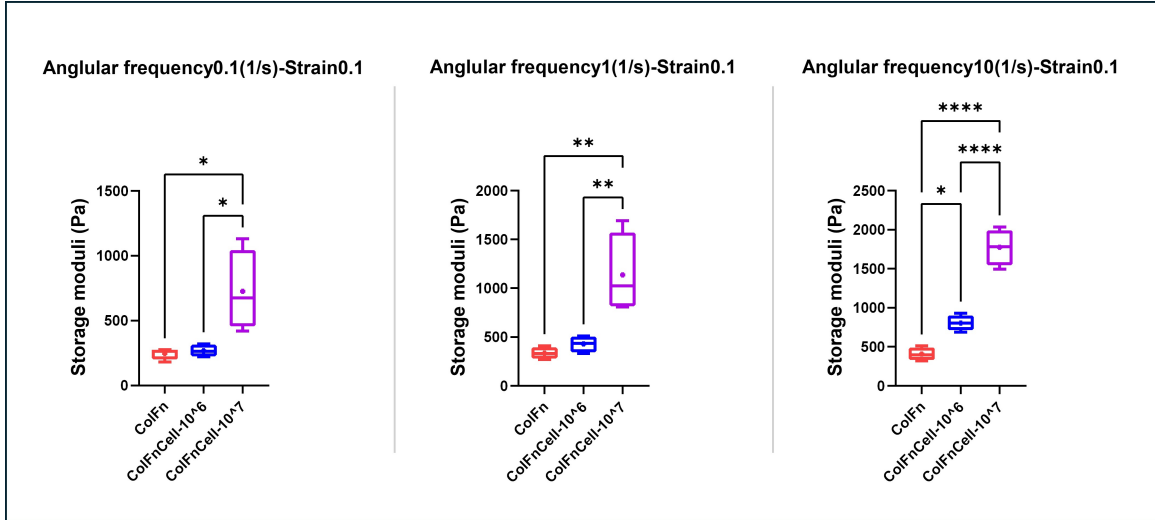


Figure 6.9: Rheological Characterization of ColFn ECM Scaffolds at Varying Cell Densities. Storage modulus of ColFn scaffolds at varying cell densities measured at angular frequencies of 0.1, 1, and 10 rad/s with a constant strain of 0.1. Increased cell density significantly enhanced ECM stiffness, particularly at higher frequencies, indicating that extensive cell-driven remodeling results in greater mechanical reinforcement.

In light of these measurements (Figure 6.9), a clear trend emerges at higher frequencies, where the storage modulus differences between the samples become more pronounced. As we can see from the figure, the highest-frequency sweep (10 rad/s) reveals the largest gap in stiffness between the ColFn scaffolds and the high-cell-density scaffolds. Because rapid deformation leaves less time for the matrix to relax or reorganize, any cell-driven reorganization, such as increased crosslink density, collagen alignment, or local stiffening, dominates the mechanical response. Consequently, the high-cell-density scaffolds, which undergo extensive changes and feature pronounced collagen fiber reorganization, exhibit substantially higher moduli under fast strain cycles. This observation aligns with prior findings, where fibroblast-mediated stiffening effects percolate through the matrix, elevating the bulk storage modulus (G') [9]. Local measurements with atomic force microscopy have shown that areas near contractile fibroblasts can be 2–3 times stiffer than surrounding regions, and this effect becomes compounded in high-density cultures, reinforcing the mechanical integrity of the entire scaffold [8]. At lower frequencies, the ECM network can partially dissipate or redistribute stresses, thereby reducing the apparent stiffness gap between samples.

Interestingly, although high cell densities also produce cavities or cracks in the ECM, the net effect of intense collagen reorganization appears to overshadow any mechanical discontinuities introduced by these voids. Thus, the rheological data support the conclusion that elevated cell concentrations not only enhance ECM stiffening overall, but also amplify the influence of matrix reorganization, particularly under more rapid deformation regimes. This stiffening has biological implications beyond mechanical properties. As the ECM becomes stiffer, cells can increase their traction forces, promoting differentiation toward myofibroblast phenotypes, similar to what occurs in fibrosis and tumor progression [10, 1]. Ehrlich's classic wound contraction studies showed that fibroblast-mediated compaction of collagen matrices enhances mechanical strength, supporting tissue closure [3]. Our system, initially soft (500 Pa), demonstrates how even normal fibroblasts can dramatically alter ECM mechanics at high density.

6.6 Conclusion

In this chapter, we investigated how high cell density fundamentally alters the structure and mechanics of ColFn ECMs. As cell density increased, we observed the emergence of distinct island-like ECM structures, enriched with Col and Fn, marking a transition from a continuous fibrous network to a clustered, compartmentalized architecture. This structural shift mirrors phenomena observed in wound healing and fibrotic tissues, where cell-driven forces reorganize the ECM into mechanically distinct domains.

Notably, this phase-transition-like reorganization was accompanied by the formation of cavities and vertical heterogeneity, arising from gravitational sedimentation during polymerization. Higher cell concentrations led to increased cell accumulation at the scaffold bottom, intensifying ECM reorganization and reinforcing structural stratification.

Our rheological analysis revealed that these structural transformations translated into a marked increase in matrix stiffness, particularly under rapid deformation conditions. This stiffening effect reflects the cumulative impact of cellular contractility, fibronectin assembly, and collagen fiber realignment, all of which reinforce the matrix's mechanical integrity. Importantly, this stiffening was not merely a passive consequence of polymerization but was actively driven by collective cell behavior, illustrating the profound influence of cell density on ECM mechanics.

These findings underscore that ECM reorganization in densely populated environments is not a gradual or uniform process but one characterized by threshold-dependent transitions, where architecture and mechanics are dramatically reshaped by collective cellular dynamics. This work provides critical insights into how high cell density governs ECM behavior, offering essential guidance for engineering tissues with controlled mechanical properties and for understanding pathological ECM reorganization processes, such as those seen in fibrosis and cancer.

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Chapter 7

General Discussion and Future Work

7.1 General Discussion

This chapter synthesizes the key findings of this thesis, demonstrating how polymerization conditions, fibronectin incorporation, and cell density collectively determine the structure, mechanics, and reorganization of ColFn hydrogels. By establishing tunable ECM platforms that capture diverse microarchitectural and mechanical environments, this work provides insights relevant to tissue repair, fibrosis, cancer progression, and broader mechanobiology applications [1, 2, 3, 4, 5, 6, 7].

The ECM is a complex and dynamic scaffold that plays a key role in directing cell behavior and tissue function. Its structure and mechanical properties provide important cues that regulate processes such as cell growth, movement, differentiation, and tissue reorganization. This thesis focuses on ColFn hydrogels, which have become essential tools in mechanobiology because they mimic the architecture and mechanical properties of natural ECM. These hydrogels are flexible platforms used to study a wide range of biological processes and diseases, including tissue repair, fibrosis, cancer progression, cardiovascular regeneration, and neuroregeneration [1, 2, 3, 4, 5, 6, 7]. Our study specifically aimed to design and characterize ColFn hydrogels with tunable microstructure and mechanics to better replicate these diverse biological environments.

In tissue repair, fibroblasts coordinate ECM deposition and reorganization, first assembling a provisional Fn network that transitions into a collagen-rich matrix to close wounds [8, 2]. The stiffness and microarchitecture of these matrices are critical: softer environments minimize scarring by permitting relaxed fibroblast behavior, whereas stiffer networks drive sustained contractility that promotes fibrosis [3, 9]. Recognizing these dynamics, we examined how adjusting polymerization conditions and incorporating Fn could modulate scaffold properties to influence fibroblast responses.

Similarly, in fibrotic diseases such as idiopathic pulmonary fibrosis, pathological stiffening arises from excessive collagen cross-linking coupled with fibroblast contractility, establishing a mechanical feedback loop that perpetuates tissue dysfunction [10].

Cancer-associated fibroblasts produce dense and aligned ColFn cables, increasing tumor stiffness and facilitating cancer cell invasion while also reducing drug delivery efficacy. This process is exacerbated by matrix heterogeneity and ongoing reorganization [11, 12, 5]. Since natural ECMs, especially in tumors, contain high cell concentrations, we aimed to model this complex environment by seeding our scaffolds at higher cell densities. This approach enabled us to explore the impact of collective cell-generated forces on ECM architecture, mimicking aspects of tumor microenvironments where mechanical changes contribute to disease progression.

Beyond pathology, ColFn hydrogels are valuable in regenerative medicine, mimicking the stiffness and ligand composition of natural ECM for cardiovascular, musculoskeletal, and nervous tissue engineering [13, 7, 6]. Moreover, ColFn hydrogels provide a platform to study how matrix stiffness influences macrophage polarization and fibroblast activation, offering insights into immuno-

mechanobiology [14, 15]. Additionally, tunable ColFn scaffolds help explore aging and ECM homeostasis by demonstrating how fibroblasts lose mechanosensitivity and how matrix organization degrades, effectively separating mechanical aging from chronological aging [16, 17]. While these areas were not directly addressed in this thesis, the ColFn scaffold system developed here offers a valuable platform for future studies exploring these processes.

The shape and mechanical properties of ColFn hydrogels, including fiber diameter, bundling, alignment, pore size, stiffness, and viscoelastic relaxation—create a mechanical language that cells interpret through integrins and force-sensitive pathways [18, 19, 20]. Each of these features plays a distinct yet interconnected role in directing cell behavior. Bundled fibers increase network tensile strength and support focal adhesion formation [21], while aligned fibers introduce directionality that guides migration and organizes force transmission [11]. Direct staining for focal adhesion proteins such as vinculin or paxillin could quantify these adhesion complexes, providing additional insight into how scaffold architecture governs cell contractility.

Pore size complements these effects by regulating the physical space available for cell and cytokine movement. Larger pores encourage mesenchymal migration modes, while smaller pores constrain movement and promote amoeboid behavior, forcing cells to adapt their shape and motility [22]. Together, fiber alignment and pore size define the microarchitecture that cells encounter within the scaffold.

In addition to these structural features, bulk stiffness plays a critical role in modulating integrin clustering and the nuclear translocation of mechanosensitive factors, which in turn influence cell fate decisions such as differentiation and contractility [18, 19]. This stiffness is not static; viscoelastic relaxation determines how long mechanical stresses persist in the matrix, affecting the timing and extent of cellular responses such as immune cell polarization and fibroblast activation [20]. While our rheometer recorded both G' (elastic) and G'' (viscous) moduli, we primarily analyzed G' because our soft hydrogels, composed of more than 90% water, exhibited low shear moduli (often below 70 Pa) where reliable G'' measurements were challenging. This reflects common limitations in rheologically characterizing highly hydrated ECM analogs, yet underscores the need for future studies with more sensitive approaches to fully capture viscoelastic contributions to mechanotransduction.

Adding to this complexity, Fn plays a key dual role in the ECM. It acts as both an adhesive ligand, providing integrin-binding sites like RGD and PHSRN, and as a mechanical regulator that reinforces the collagen network and organizes fibrils [23, 24]. The dynamic interplay between Col and Fn—where Fn tensions Col fibers and Col stabilizes Fn assemblies, creates an integrated biochemical and mechanical signaling system that cells continually interpret and remodel [24]. In this thesis, we leveraged these interconnected structural and mechanical principles to systematically investigate how polymerization conditions and Fn incorporation modulate ColFn scaffold properties. By doing so, we created ECM models that more accurately reflect the complexity and dynamism of natural tissues, and we explored how these properties direct cell behavior under both physiological and pathological conditions.

These properties are crucial for replicating the dynamic environments found in living tissues. In tissue repair, adjusting fiber thickness and viscoelasticity can limit hypertrophic scarring and accelerate re-epithelialization [25]. In fibrosis, modifying scaffold stiffness recreates the progression from soft to stiff tissue, aiding drug screening for treatments that can counteract fibrosis [21, 26]. In cancer, variations in mechanical stiffness guide carcinoma cells along stiffness gradients, promoting invasion [11, 12], while Fn clusters create pathways and specialized biochemical environments that facilitate cancer cell invasion [27]. Our observation of phase transitions and ECM stiffening at high cell densities offers insights into how mechanical environments may contribute to disease progression.

Traditional ECM models such as Col-only have important limitations. They maintain static stiffness, have simple compositions, and lack the dense, dynamic environments found in real tissues [28, 29, 30]. Most of these models do not allow for adjustable viscoelasticity or controlled fiber alignment, and they often fail to replicate the phase transitions and protein clustering driven by cells in living tissues [31, 32, 33]. Variability between batches and poor reproducibility further limit their use in precise mechanobiology studies [34]. This thesis overcame these challenges by using temperature-controlled casting to create scaffolds with a physiologically relevant stiffness range, systematically incorporating Fn to increase biochemical complexity and mechanical strength,

and exploring cell densities up to 10^7 cells/mL to observe emergent phase transitions and ECM reorganization behaviors.

The integration of live-cell imaging, oscillatory rheology, and confocal microscopy allowed dynamic tracking of ECM formation, reorganization, and local mechanical variations, offering a robust platform that better represents real tissue environments and advancing mechanobiology research and regenerative medicine applications [35, 32, 36].

The following sections provide detailed experimental results supporting these ideas, including comparisons between cold cast and warm cast hydrogels, the role of Fn in shaping ECM structure and mechanics, and how high cell density influences ECM organization and phase transitions.

7.2 Key Experimental Findings and Analysis

This work establishes a controlled and reproducible method to tune both the microstructure and mechanical properties of collagen–fibronectin hydrogels, enabling a systematic investigation of Dynamic Reciprocity between fibroblasts and their extracellular matrix. By altering polymerization temperature, incorporating fibronectin, and varying cell density, we generated scaffolds in which structural and mechanical parameters can be independently or jointly modulated. Cold and warm cast collagen scaffolds exhibited markedly different microarchitectures while displaying similar bulk stiffness, whereas the addition of fibronectin increased stiffness and amplified structural differences between the two polymerization conditions. Using these engineered ECMs, we quantified how fibroblasts actively reorganize their environment and how ECM architecture, stiffness, and biochemical composition shape cell behaviour. In the presence of cells, the ECM underwent continuous structural evolution (including fiber rearrangement, alignment, and localized collagen and fibronectin island formation) whereas acellular scaffolds remained stable and matured slowly. We further found that fibroblasts drive global stiffening or softening depending on network connectivity. Warm cast, highly connected networks stiffened uniformly, while cold cast, weakly connected networks softened despite local alignment. Pair-distribution function and heterogeneity analyses revealed that these contrasting behaviors arise from differences in how cell-generated forces propagate through networks with distinct connectivity. At high cell densities, we identified a previously unreported density-driven phase transition in which continuous networks collapse into discrete ColFn islands accompanied by strong cell-mediated stiffening. Together, these findings define how engineered ECM design and cellular activity converge to regulate 3D matrix organization and mechanics.

Our work began with the fabrication of collagen hydrogels using two distinct polymerization approaches. Cold cast scaffolds, polymerized gradually from 4°C to 37°C , yielded thicker fibers ($3.4 \pm 0.35 \mu\text{m}$), longer fibers ($52.9 \pm 7.9 \mu\text{m}$), and larger pores ($2541.6 \pm 1375.4 \mu\text{m}^2$). In contrast, warm cast scaffolds, polymerized directly at 37°C , produced thinner ($2.25 \pm 0.23 \mu\text{m}$), shorter ($38.2 \pm 4.4 \mu\text{m}$) fibers with smaller pores ($1797.6 \pm 1003.5 \mu\text{m}^2$). Despite these structural differences, both scaffold types exhibited comparable bulk stiffness at 10 s^{-1} ($842.5 \pm 244.2 \text{ Pa}$ for cold cast and $604.5 \pm 361.4 \text{ Pa}$ for warm cast), providing two distinct yet mechanically equivalent platforms for studying cell behavior. These results demonstrate that polymerization temperature can tune microstructure without necessarily altering bulk stiffness, allowing structural and mechanical contributions to be evaluated independently in cell–ECM studies.

To modulate ECM formation and biochemical interactions, Fn was incorporated at 0.2 mg/mL . This addition further reduced fiber diameters and pore sizes: cold cast with Fn yielded fibers of $2.63 \pm 0.29 \mu\text{m}$, lengths of $36.5 \pm 5.0 \mu\text{m}$, and pores of $1453.2 \pm 460.6 \mu\text{m}^2$; warm cast with Fn produced fibers of $1.66 \pm 0.14 \mu\text{m}$, lengths of $28.4 \pm 3.0 \mu\text{m}$, and pores of $593.4 \pm 170.9 \mu\text{m}^2$. Rheology confirmed that Fn incorporation enhanced bulk stiffness to $918 \pm 176.1 \text{ Pa}$ for cold cast and $1150 \pm 96.7 \text{ Pa}$ for warm cast samples. Fn provided additional adhesion sites via its FNII and FNI subunits and interacted with integrins, particularly $\alpha_5\beta_1$, through RGD and PHSRN motifs, establishing biochemical links that enhanced cell–ECM interactions. Thus, fibronectin strengthens the network and amplifies microstructural differences between casting conditions, providing an additional axis for tuning ECM mechanics.

To understand how these microarchitectural differences influence cellular reorganization, we per-

formed pair-distribution function (PDF) analysis. Warm cast scaffolds displayed a shallow, near-linear $g(r)$ decay, consistent with a dense, continuous network that distributes forces evenly. Cold cast scaffolds exhibited a curved $g(r)$ profile, reflecting fiber clustering and lower connectivity. Upon fibroblast seeding, warm cast $g(r)$ curves changed minimally, whereas cold cast scaffolds showed a pronounced shift, indicating local fiber realignment and bundle formation. These results show that network connectivity, rather than fiber thickness alone, determines how cell-generated forces propagate through the ECM.

Rheological measurements across angular frequencies (0.1, 1, 10 rad/s) demonstrated that stiffening or softening effects were frequency-dependent. At lower frequencies, differences between conditions were less pronounced; at higher frequencies (10 rad/s), cell-mediated stiffening in warm cast and softening in cold cast scaffolds became more apparent. This highlights the importance of dynamic mechanical testing for capturing cell-mediated ECM reorganization.

Expanding from these mechanical characterizations, we conducted live-cell imaging to examine ECM formation kinetics and cell dynamics during hydrogel polymerization at collagen concentrations of 2.88 and 3.69 mg/mL. Higher collagen concentration (3.69 mg/mL) accelerated fiber formation, reducing half-max times to $t_{1/2} \approx 3.4$, 4.6, and 2.6 min for nucleation, thickening, and lengthening, respectively, compared to 8.4, 9.7, and 6.1 min for 2.88 mg/mL. Exponential rate constants also increased (e.g., $k_N = 0.206 \text{ min}^{-1}$ for 3.69 mg/mL vs. 0.083 min^{-1} for 2.88 mg/mL), and Fn further enhanced assembly speed ($t_{1/2} \approx 6.3 \text{ min}$). Live imaging revealed dynamic pericellular compaction, transient collagen islands, persistent fibronectin islands, and z-axis cell migration leading to gravitational sedimentation, which created vertical heterogeneity with denser cell populations at the scaffold bottom.

At higher cell densities (10^6 – 10^7 cells/mL), the ECM exhibited a qualitatively different mode of reorganization. Microscopy revealed a density-driven phase transition occurring above approximately 5×10^6 cells/mL, in which the continuous fibrillar network fragmented into discrete Col, Fn islands separated by cell-free cavities. Surrounding fibroblasts generated contractile forces that realigned fibers along the cavity boundaries, producing patterns reminiscent of early wound-healing responses. Bulk rheology measurements confirmed substantial stiffening at higher frequencies, consistent with collective force generation and enhanced mechanical reinforcement of the matrix. These findings identify a previously unrecognized, density-dependent structural transition in ColFn hydrogels, highlighting how collective cell mechanics can reorganize ECM architecture at multicellular scales.

7.3 Conclusion

This thesis provides a comprehensive framework for understanding how scaffold fabrication methods, fibronectin incorporation, and cell density collectively determine ECM structure, mechanics, and reorganization in ColFn hydrogels. These insights offer new avenues for engineering physiologically relevant ECM models.

We established that polymerization kinetics govern scaffold microarchitecture, producing distinct platforms (cold cast vs. warm cast) with comparable bulk stiffness but contrasting fiber organization. The incorporation of fibronectin further stabilized and refined these networks, enhancing mechanical strength and providing integrin-mediated binding sites that redirected cell–matrix interactions.

Furthermore, our findings demonstrate that cells actively reshape the ECM, with outcomes influenced by initial scaffold structure and biochemical composition. In dense networks, cells increased stiffness globally, whereas in discontinuous scaffolds, cell-generated forces disrupted connectivity, causing overall softening. These results, supported by quantitative PDF analysis and rheology, underscore the mechanistic link between scaffold architecture and cellular behavior.

At higher cell densities, we observed a threshold-dependent phase transition, where fibrous networks transformed into discrete Col, Fn islands with fiber alignment in surrounding cell-free cavities. This behavior, akin to wound healing and cancer-associated stiffening, underscores the profound impact of collective cell dynamics on ECM architecture and mechanics.

Overall, this thesis highlights the dynamic interplay between scaffold design, biochemical modifi-

cation, and cellular behavior. Our findings provide valuable insights for engineering biomimetic ECM platforms with tunable properties, offering promising applications in tissue engineering, regenerative medicine, and disease modeling.

7.4 Main Limitations of This Work

While this thesis establishes a controlled and tunable platform for studying cell–ECM interactions in ColFn hydrogels, several limitations should be acknowledged.

First, although cellular contractility and cell-driven matrix reorganization are central to many of the observed phenomena, including ECM stiffening, fiber alignment, and phase-transition behavior at high cell densities, no direct measurements of cell–ECM traction forces were performed. Techniques such as traction force microscopy or detailed focal adhesion imaging were not implemented. As a result, cell-generated forces were inferred indirectly from structural reorganization and rheological stiffening rather than measured explicitly.

Second, ECM architecture and mechanics evolved simultaneously throughout the experiments. Polymerization conditions, fibronectin incorporation, and cell-mediated remodeling altered both microstructure and stiffness in parallel. While this coupling reflects the behavior of native tissues, it complicates direct mechanistic separation of purely structural effects from mechanical feedback. Although pair-distribution function analysis and rheology provided complementary insight, disentangling these coupled effects remains an inherent limitation of the current experimental design.

Third, ECM remodeling was primarily assessed through endpoint imaging and bulk mechanical measurements. While confocal microscopy revealed substantial reorganization, clustering, and cavity formation, this approach does not directly quantify *de novo* collagen or fibronectin deposition, matrix degradation, or enzymatic remodeling by matrix metalloproteinases (MMPs). Consequently, the biochemical balance between ECM synthesis and degradation could not be explicitly resolved.

Fourth, an additional limitation arises from incomplete control over cell distribution along the vertical (z) direction of the scaffolds. Although a sample-flipping strategy was implemented during early incubation to mitigate gravitational sedimentation and improve cellular uniformity, fully homogeneous cell distribution throughout the scaffold thickness could not be achieved. This limitation became increasingly pronounced at higher cell densities, particularly at 10^7 cells/mL, where significant differences between the top and bottom regions of the samples were consistently observed. As a result, local variations in ECM reorganization, clustering, cavity formation, and mechanical response reflect not only density-dependent cellular activity but also sedimentation-driven stratification. Future studies could overcome this limitation through approaches such as rapid pre-gelation viscosity enhancement, rotational or clinostat-based culture or microfluidic encapsulation strategies to achieve more uniform three-dimensional cell distributions.

Finally, the biological model employed here represents a simplified approximation of native tissue environments. The use of mouse fibroblasts within a two-component collagen–fibronectin ECM does not capture the full cellular, biochemical, and mechanical complexity of native tissues, which include multiple stromal and immune cell populations, additional ECM components, and soluble biochemical factors. While this reductionist approach enables precise mechanistic control, it limits direct extrapolation to fully *in vivo* settings.

Together, these limitations do not diminish the central findings of this work but instead define important boundaries for their interpretation and highlight opportunities for future investigation.

7.5 Outlook and future work

Building on the foundational insights developed in this thesis, several promising research directions offer opportunities to significantly advance the understanding and applications of ColFn hydrogels as biomimetic ECM platforms. ColFn ECMs have become valuable 3D culture platforms, offering a biomimetic ECM environment for both tissue engineering and tumor microenvironment studies [35, 36].

To further capture dynamic ECM reorganization, future studies should expand live-cell imaging to incorporate advanced three-dimensional techniques, such as lattice light-sheet and two-photon microscopy. These methods offer real-time visualization of cell behavior and ECM reorganization deep within scaffolds while minimizing phototoxicity [37].

Based on this, integrating imaging setups directly within incubators could enable longer-term observation of cell dynamics under physiological conditions. This approach would provide insights into phenomena such as matrix invasion, cell migration along stiffness gradients, and detailed cell–ECM interactions that are difficult to observe in traditional 2D culture systems [35, 37].

In parallel with imaging approaches, incorporating detailed analyses of the cytoskeleton and cell adhesion structures would further illuminate how cells respond to ECM architecture. Techniques such as actin staining or genetically encoded reporters like LifeAct-GFP can reveal how cells organize their cytoskeleton in response to three-dimensional matrix cues [38]. Equally important, immunostaining for focal adhesion components—including vinculin, paxillin, or phosphorylated FAK—would allow quantification of the number, size, and elongation of adhesion complexes. These parameters provide direct readouts of cell–matrix traction, adhesion strength, and contractile behavior [39, 40]. For example, larger and more elongated focal adhesions typically indicate stronger integrin-mediated attachments and higher tension transmission [39, 41]. Integrating these analyses would have complemented our current studies by directly correlating scaffold mechanical and structural features with individual cell–ECM interactions, offering a finer-grained understanding of how matrix mechanics modulate cell contractility and adhesion dynamics.

To deepen the understanding of ECM mechanics, future studies should integrate atomic force microscopy (AFM)-based force mapping with bulk rheology [42, 43]. AFM can reveal localized stiffness variations between individual fibers and pores, offering a more detailed view of the micro-scale mechanical heterogeneity within ColFn scaffolds. By combining these local stiffness maps with real-time cell imaging, researchers can correlate mechanical cues with observed cellular responses. This integrated approach would enhance scaffold design for applications in tissue engineering and cancer modeling [44, 45].

Expanding further, exploring combinatorial ECM scaffolds with controlled variations in composition, architecture, and stiffness represents a promising research direction. Engineered interfaces, such as distinct collagen–fibronectin regions or stiffness gradients, can mimic physiological scenarios like tumor-stroma boundaries or wound edges [35, 32]. Studying how cells behave across these interfaces can provide valuable insights into cell sensing mechanisms, migration patterns, and ECM reorganization, paving the way for more effective scaffold designs for regenerative medicine and disease modeling [32, 35].

Integrating advanced computational modeling with experimental studies will be essential for developing predictive frameworks of scaffold assembly and cell–ECM interactions. Finite-element and multi-scale modeling approaches can simulate cell traction forces, ECM deformation, and scaffold mechanical responses at cellular and molecular levels [46, 47, 48]. By refining these models through experimental validation, researchers can accelerate scaffold optimization and deepen mechanistic insights into ECM dynamics. Computational models also offer tools to test hypotheses, simulate complex scenarios such as heterogeneous ECM interfaces, and predict long-term reorganization outcomes that are challenging to observe experimentally [46, 49].

Pursuing these research directions will significantly advance the fundamental understanding of ECM dynamics and cellular mechanobiology within ColFn scaffolds. By leveraging cutting-edge imaging, mechanical characterization, ECM interface engineering, and computational modeling, future studies can more effectively translate these insights into practical applications. These efforts promise not only to deepen mechanistic insights into ECM dynamics but also to inform the development of sophisticated biomimetic platforms for regenerative medicine, improve disease modeling strategies, and ultimately pave the way for next-generation therapies addressing tissue repair, fibrosis, and cancer progression [35, 32, 36].

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Appendix

Comparative Analysis of ColCell vs. ColFnCell: Fibronectin's Effect in Presence of Cells

It can be observed from Figure A1 and table A1 that the addition of fibronectin to collagen scaffolds in the presence of cells has a significant impact on the microstructure and rheological properties of the scaffolds. The results show that the average collagen fiber diameter, length, and pore/mesh size of the *ColFnCell* scaffolds are smaller than those of the *ColCell* scaffolds.

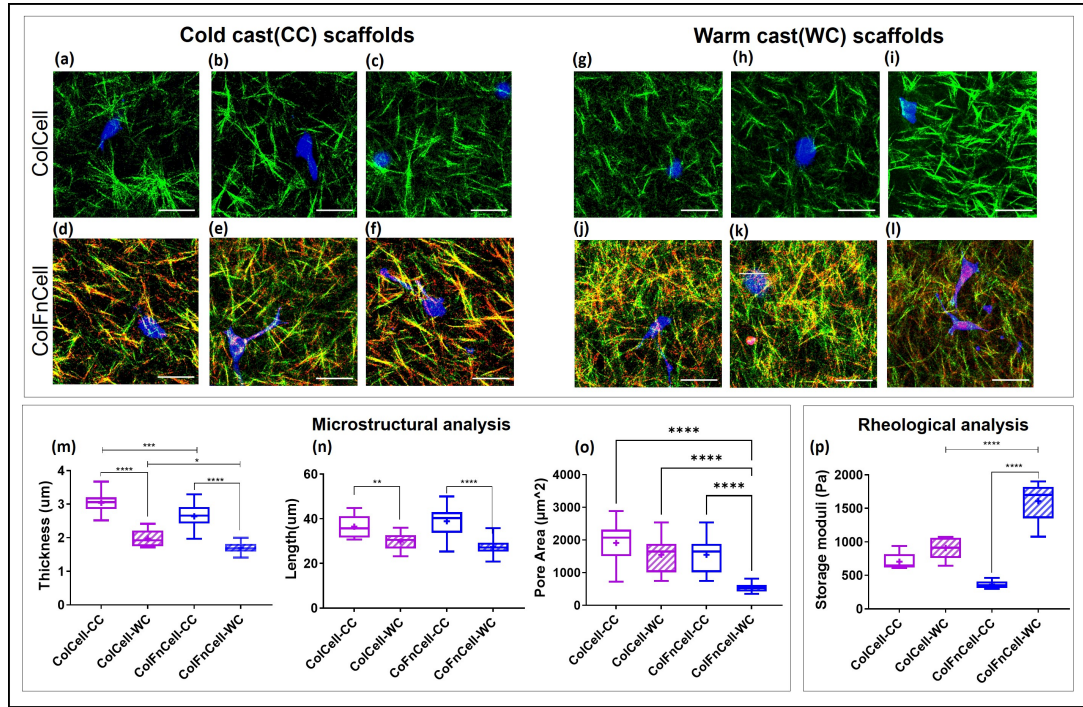


Figure A1: Microstructure and rheological analysis of cell-laden Collagen and Collagen-Fibronectin scaffolds. (a-c) Cold-cast collagen-cell (*ColCell-CC*). (d-f) Cold-cast collagen-fibronectin-cell (*ColFnCell-CC*). (g-i) Warm-cast collagen-cell (*ColCell-WC*). (j-l) Warm-cast collagen-fibronectin-cell (*ColFnCell-WC*). (m-o) Quantification of fiber diameter, fiber length, and pore/mesh area for each scaffold type. (p) Storage modulus G' measured at 0.1% strain and 10 s^{-1} ($n = 4$). Scale bar: 50 μm .

This suggests that the presence of fibronectin in the scaffolds enhances the cell attachment and infiltration, resulting in a more compact and dense structure. It has been proven that fibronectin can form a complex network of fibrils that can interact with collagen fibers and regulate the assembly of the ECM. This interaction between fibronectin and collagen fibers can lead to the formation of a more organized and denser microstructure, which can improve the cell adhesion and proliferation

on the scaffold [1].

Table A1: Structural and Rheological Properties of ColCell and ColFnCell Scaffolds

Scaffold Type	Fiber Diameter (μm)	Fiber Length (μm)	Pore Size (μm^2)	Dynamic Shear Moduli (Pa)
ColCell-CC	3.04 $\pm$ 0.29	36.5 $\pm$ 5.0	1908 $\pm$ 591	704 $\pm$ 134
ColCell-WC	1.97 $\pm$ 0.22	29.8 $\pm$ 3.8	1546 $\pm$ 537	909 $\pm$ 172
ColFnCell-CC	2.63 $\pm$ 0.35	38.9 $\pm$ 6.7	1546 $\pm$ 519	361 $\pm$ 61
ColFnCell-WC	1.69 $\pm$ 0.15	27.4 $\pm$ 4.1	584 $\pm$ 115	1608 $\pm$ 280

Additionally, the dynamic shear moduli of the *ColFnCell* scaffolds are higher than those of the *ColCell* scaffolds, indicating that the presence of fibronectin enhances the mechanical properties of the scaffolds. In addition to the above effects, the presence of cells in the scaffolds also plays a crucial role in altering the rheological properties of the *ColFnCell* scaffolds. It has been observed that the storage modulus, which is a measure of the stiffness of the scaffolds, is significantly increased in the presence of cells. This suggests that the cells contribute to the mechanical properties of the scaffolds by stiffening the samples further. However, even in the absence of cells, the addition of fibronectin can still have an effect on the collagen ECM, as it can interact with collagen fibers and regulate their assembly to form a more organized and denser microstructure.

Structural Analysis of Fibronectin Fibers

The results from the fiber thickness and length analyses, presented in Figure A2, reveal a strong dependency of fibronectin fiber characteristics on the casting temperature conditions within collagen-based scaffolds. The box plots clearly illustrate statistically significant differences in both fiber thickness and fiber length between CC and WC samples.

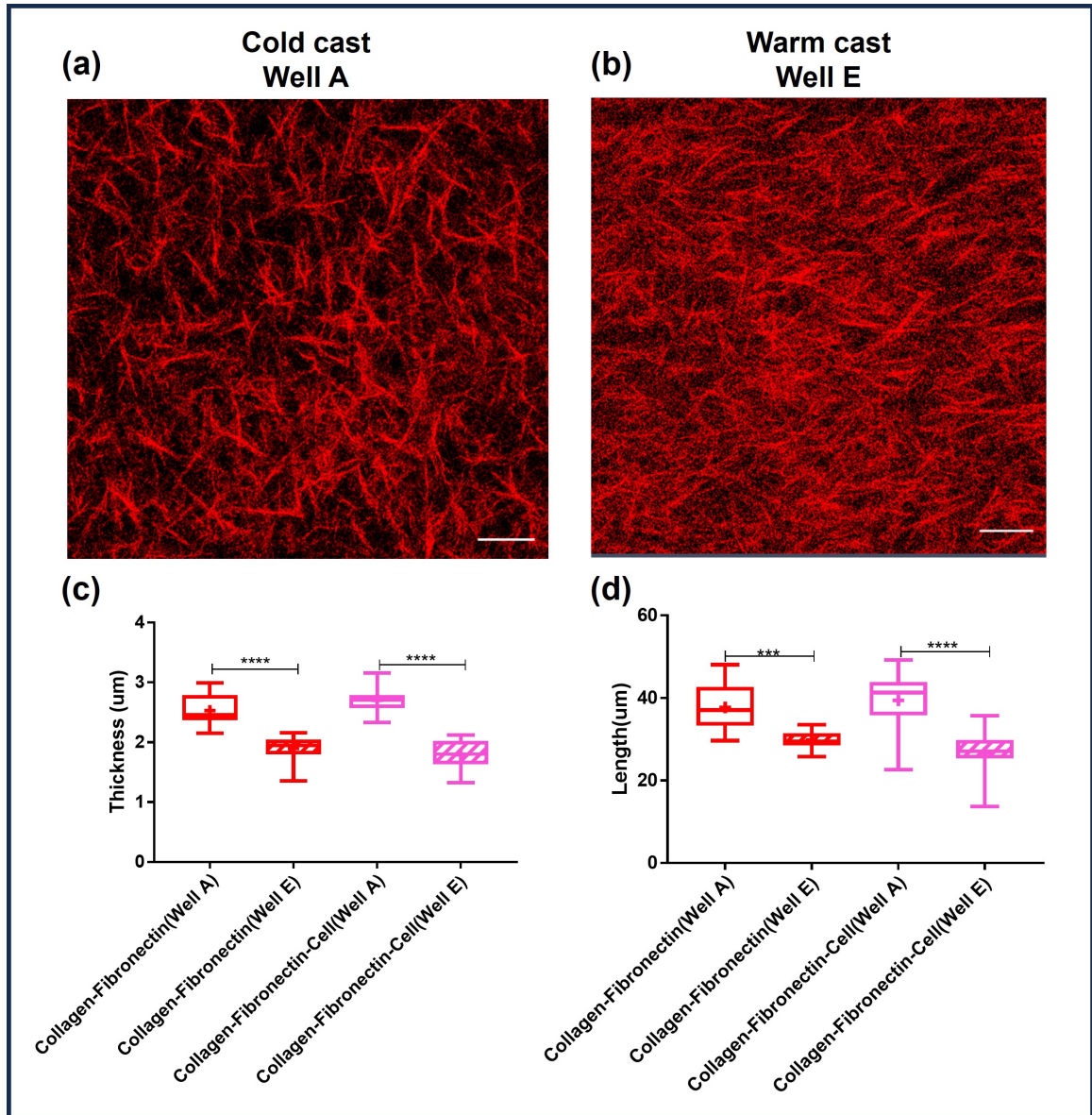


Figure A2: Microstructural analysis of Fn fibers. Confocal microscopy images (red: Fibronectin) comparing the fibronectin fiber structure in: a) CC and b) WC scaffolds.

For fiber thickness, highly significant differences (****) were detected between the ColFn-CC and ColFn-WC groups, as well as between the ColFnCell-CC and ColFnCell-WC groups, confirming that warm casting consistently promotes the formation of thinner fibronectin fibers, irrespective of cellular presence.

Regarding fiber length, significant differences (*) were found between ColFn-CC and ColFn-WC scaffolds, while highly significant differences (**) emerged when comparing ColFnCell-CC with ColFnCell-WC scaffolds. These results suggest that warm casting notably shortens fibronectin fibers, an effect amplified in the presence of cells.

Interestingly, the observed trends in fibronectin fiber structure closely mirror those previously observed for collagen fibers. Overall, these data highlight that higher casting temperatures consistently lead to the formation of thinner and shorter fibronectin fibers within collagen scaffolds, emphasizing the critical role of thermal conditions in defining ECM microstructural properties.

Additional Frequency-Sweep Data

In this appendix, we present the additional frequency-sweep data for the storage modulus G' measurements that were mentioned in Chapter 4. These results complement the discussion by showing how the mechanical responses vary across multiple frequencies.

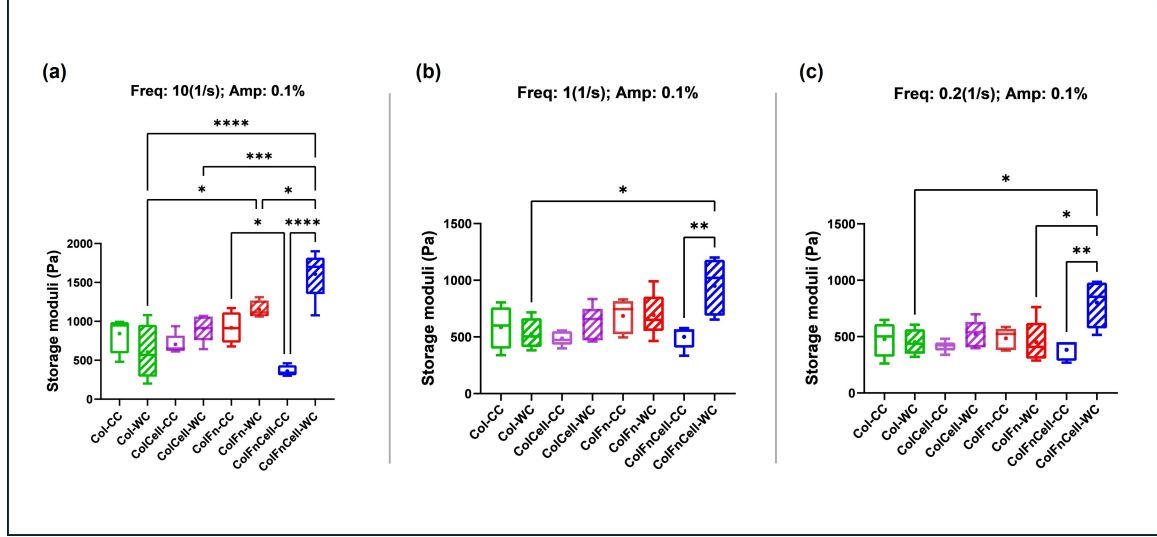


Figure A3: Comparison of storage moduli at multiple frequencies (0.1 s^{-1} , 1 s^{-1} , and 0.2 s^{-1}) for the various hydrogel samples. This figure complements the data in Chapter 2, showing how G' varies at additional test frequencies.

Figure A3 presents box plots of G' measured at multiple frequencies for all the samples. In this plot, samples prepared by cold casting or warm casting are compared separately and the difference between cold cast and warm cast samples ignored; the only exception is one case where the samples differed solely by the casting method (cold cast versus warm cast) while maintaining identical other parameters.

Figure A3 displays the storage modulus G' measured at different frequency for a range of collagen-based hydrogel samples. In this plot, only samples prepared under similar casting conditions are compared directly; that is, cold cast samples are compared only with other cold cast samples and warm cast samples only with other warm cast samples (except when the material is identical, such as between Col-CC and Col-WC).

In Figure A3 a) significant differences between sample pairs are indicated by star symbols: A two-star (**) difference is observed between the *ColFnCell-WC* and *ColFnCell-CC* groups. A one-star (*) difference is noted between *Col-WC* and *ColFnWC*.

These differences are discussed in detail in Chapter 4, with the sole exception of the *Col-WC* vs. *ColFnCell-WC* comparison, which was intentionally omitted from the chapter discussion because it is self-explanatory.

Furthermore, a comparison of parts (a), (b), and (c) of the figure reveals that as the testing frequency decreases, the mechanical differences between the samples become less pronounced. Specifically, in part (b), only two pairwise differences remain statistically significant: one between *ColFnCell-WC* and *ColFnCell-CC* (indicated by two stars), and another between *Col-WC* and *ColFn-WC* (one star). At very low frequency (part (c)), the significant differences are similarly limited, appearing only between *ColFnCell-WC* and *ColFnCell-CC* (two stars), between *Col-WC* and *ColFn-WC* (one star), and between *ColFn-WC* and *ColFnCell-WC* (one star).

These results suggest that at lower frequencies, where the polymer network has more time to dissipate energy, the differences in stiffness among the various hydrogels tend to converge. This convergence highlights the increasing contribution of viscous processes to the overall mechanical response at low frequencies.

Frequency and Amplitude-Dependent Mechanical Behavior of Collagen-Based Scaffolds

To ensure that all rheological measurements were performed within the linear viscoelastic region (LVR), amplitude (strain) sweeps were conducted for each scaffold type prior to frequency and time-dependent testing (Figure A4). The storage modulus G' remained constant up to approximately 0.6% strain across all samples, indicating a well-defined LVR. A strain amplitude of 0.1% was therefore selected for all subsequent measurements. For completeness, frequency sweep data showing the storage modulus G' as a function of angular frequency are also included in this appendix (next section). All measurements were averaged over at least five replicates for each scaffold type.

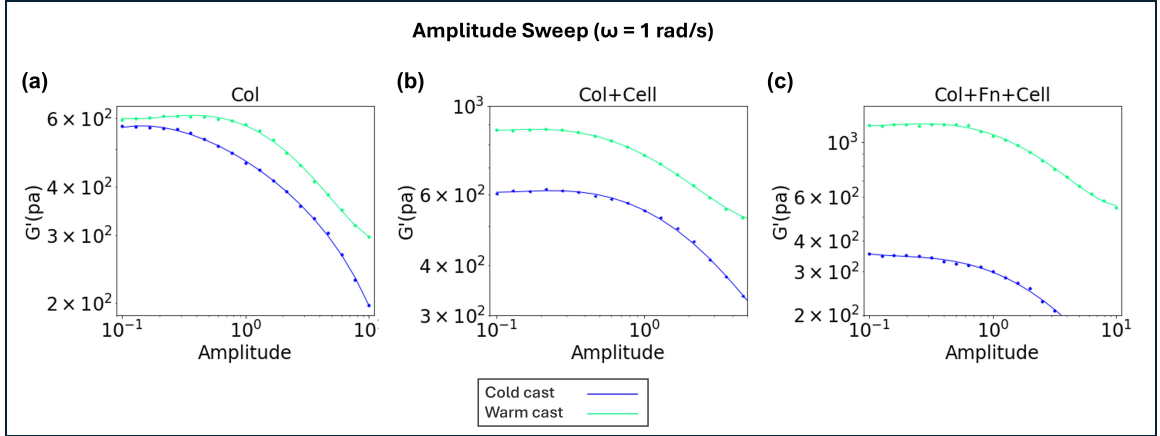


Figure A4: Amplitude-dependent storage modulus (G') of collagen-based scaffolds at a constant frequency of 1 (rad/s). (a) Pure collagen scaffold, (b) collagen scaffold with embedded cells, and (c) collagen-fibronectin scaffold with embedded cells. The plots represent the variation in storage modulus (G') as a function of amplitude (log scale), with measurements taken at different samples (CC: blue, WC: green). The results confirm that all samples were tested within the linear viscoelastic region (LVR). The storage modulus (G') remained constant up to approximately 0.6% strain for all scaffold compositions, indicating a well-defined LVR. Accordingly, all frequency-sweep measurements reported here were performed within the safe linear regime.

The storage modulus (G') data presented in Figure A5 highlights how scaffold stiffness varies as a function of frequency for different sample compositions. A key observation from these plots is the convergence of storage moduli at lower frequencies, indicating that mechanical differences between scaffolds become less pronounced under slow deformation. This suggests that at equilibrium-like conditions, where external forces are applied over longer timescales, the network structure of the scaffolds dominates the mechanical response, minimizing differences between sample groups.

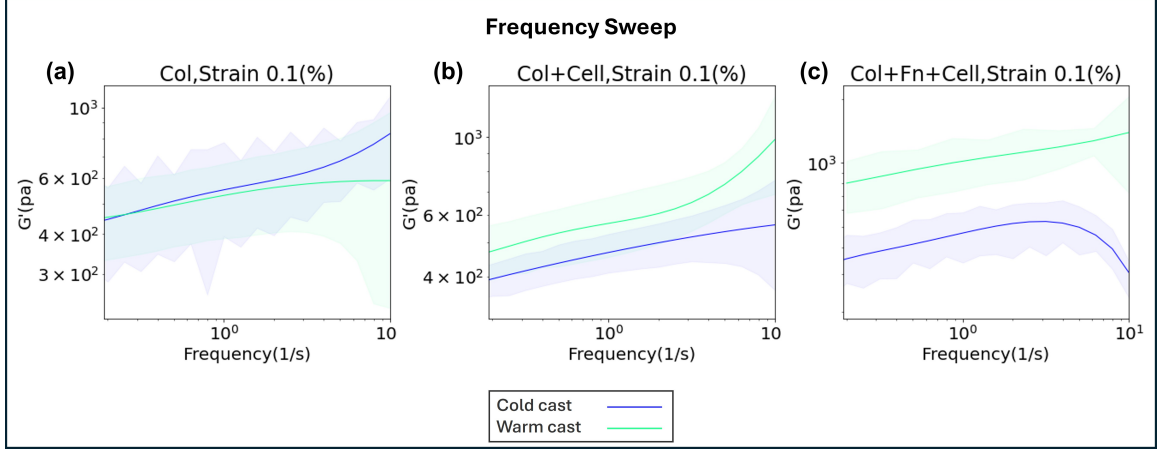


Figure A5: Frequency-dependent storage modulus (G') of collagen-based scaffolds at a constant strain amplitude of 0.1%. (a) Pure collagen scaffold, (b) collagen scaffold with embedded cells, and (c) collagen-fibronectin scaffold with embedded cells. The plots represent the variation in storage modulus (G') as a function of frequency (log scale), with measurements taken at different samples (CC: blue, WC: green). Shaded regions indicate the variability in measurements. The results demonstrate how scaffold composition and cellular incorporation influence the viscoelastic behavior of the extracellular matrix, with fibronectin-modified scaffolds exhibiting distinct mechanical responses at varying frequencies. The presence of fibronectin and cells leads to notable differences in stiffness and frequency dependence, particularly at higher frequencies.

In contrast, at higher frequencies, distinct mechanical behaviors emerge, more clearly demonstrating strain-dependent stiffening and softening effects. These nonlinear responses indicate that structural features of the scaffolds, such as fiber entanglements, crosslinks, and interactions between collagen and fibronectin, become more pronounced under dynamic loading [2]. Strain stiffening, observed in some cases, suggests the recruitment of additional load-bearing elements within the ECM, while strain softening reflects network disruptions or fiber realignment under rapid deformation.

Notably, scaffolds containing both fibronectin and cells (Figure A5c) exhibit more complex frequency-dependent behavior compared to collagen-only (Figure A5a) or collagen-cell samples (Figure A5b). This suggests that fibronectin plays a role in regulating scaffold mechanics, particularly at higher frequencies where transient cellular and ECM interactions become more significant.

Overall, these findings underscore the importance of frequency-dependent analysis in understanding scaffold mechanics. While low-frequency measurements reflect the baseline structural properties of the ECM, high-frequency responses uncover hidden mechanical adaptations that are otherwise undetectable under static conditions. This behavior has critical implications for scaffold design, particularly in applications where cells are exposed to dynamic mechanical forces, such as tissue engineering and regenerative medicine.

Image analysis

The `Stack` function (Figure A6) was designed to merge multiple grayscale images into a single RGB composite, highlighting their differences and overlaps using distinct colors. This approach ensures that structural variations across images are visually distinguishable.

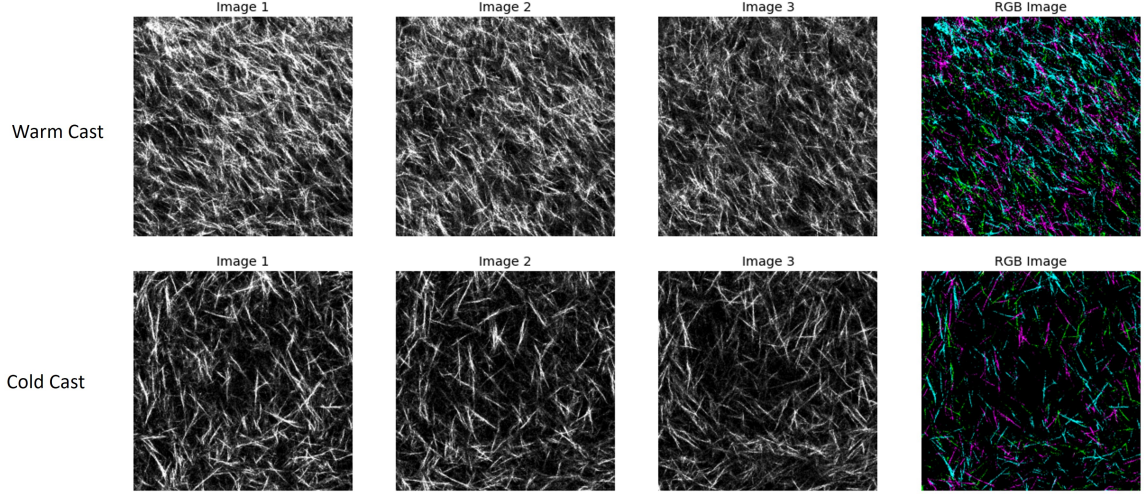


Figure A6: Stacked Image Analysis. The figure illustrates the stacking function where three layers are merged. Each image is converted to grayscale and assigned a unique color representation. The final stacked image is generated by combining these processed layers, effectively visualizing structural differences across the input images. The image dimensions are $400 \mu\text{m} \times 400 \mu\text{m}$.

The Stack function serves as a powerful tool for combining multiple grayscale images into a single, color-coded visualization, enabling a clearer interpretation of structural overlaps and variations. This method enhances the ability to analyze patterns and relationships within image datasets.

The `process_image` function (Figure A7) was designed to analyze grayscale images by detecting fiber structures, extracting key properties, and computing spatial relationships between fibers. The function performs a sequence of image processing steps, ultimately generating insights about fiber orientation and pairwise distances. **Gaussian blur**, thresholding, and morphological transformations such as **opening** and **closing** are applied to the output image to remove noise and fill gaps in the image. The thresholding is performed using automatic global thresholding algorithms (`cv2.THRESH_OTSU`).

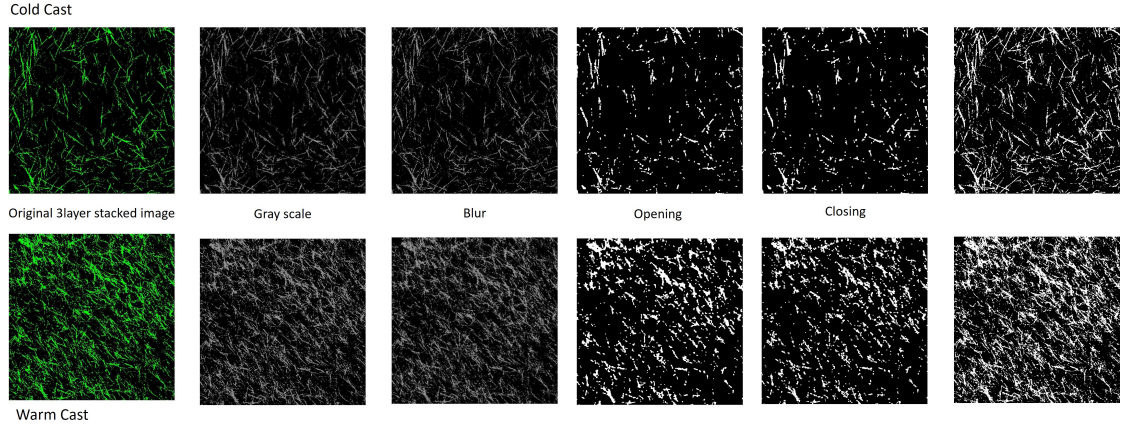


Figure A7: Morphological Processing of Fiber Structures. Comparison of Cold Cast and Warm Cast fiber structures through sequential image processing steps, including grayscale conversion, *Gaussian blur*, thresholding (`cv2.THRESH_OTSU`), and morphological transformations (*opening* and *closing*). Image dimensions: $400 \mu\text{m} \times 400 \mu\text{m}$.

In the next step, once the fibers are well-defined, we contoured aggregated and separated fibers using `cv2.findContours`. A minimum area threshold was defined to remove the effect of fiber tails.

For each detected fiber, key properties such as center of mass (used to determine fiber position by using `cv2.moments()`), convex hull (a simplified shape around the fiber by using `cv2.arcLength()`), and Length and orientation (using

ellipse fitting) are computed and stored in a DataFrame, allowing structured analysis.

From the center of each contour to the convex hull, individual fibers were distinguished. Each individual fiber was then divided into smaller circular segments, and their information, including location and labels, was saved as a CSV file (Figure A8). To analyze the spatial distribution of fibers, pairwise distances between fiber centers are calculated by setting `distance = np.linalg.norm(center1 - center2)`.

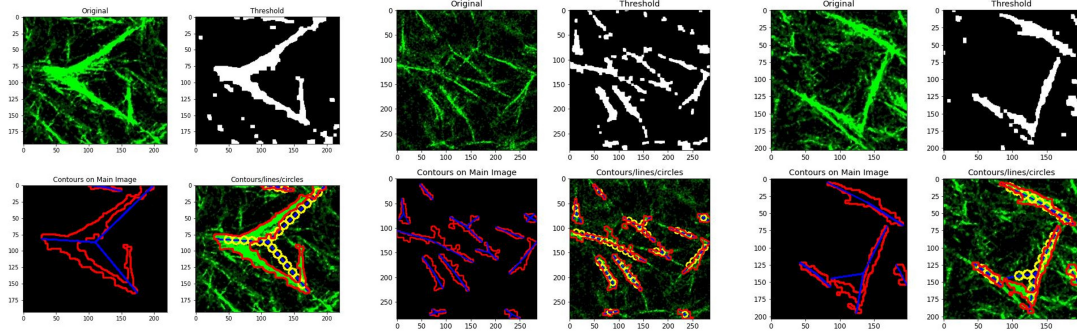


Figure A8: Fiber Detection and Contour Analysis. *Illustration of the step-by-step fiber detection process. The original image is thresholded to extract fiber structures, followed by contour detection using `cv2.findContours`. Identified contours are further analyzed to extract key properties, including the center of mass (using `cv2.moments()`), convex hull (using `cv2.arcLength()`), and fiber orientation (via ellipse fitting). Individual fibers are segmented into smaller circular regions, and their locations are labeled and saved as a CSV file. Pairwise distances between segmented fiber regions are calculated and stored separately. Image dimensions: $100\ \mu\text{m} \times 100\ \mu\text{m}$.*

To study the spatial arrangement of fibers, the function computes a histogram of pairwise distances, then normalizes distances using shell volumes, and finally plots the Pair Distribution Function (PDF), which reveals structural patterns in fiber distribution.

This script processes a set of grayscale images, extracts fiber properties, and computes pairwise distances to analyze fiber distributions in different datasets. By applying image processing techniques, such as thresholding, morphological operations, and contour detection, the script effectively identifies fibers and measures their spatial relationships.

The computed pairwise distances provide a quantitative assessment of fiber clustering and separation within each dataset. The PDF further characterizes these distances, helping to identify patterns in fiber spacing. Additionally, visual representations of the processed images allow for qualitative validation of the extracted fiber structures.

This workflow offers a structured approach to fiber analysis, enabling comparisons between datasets based on measurable spatial properties. The generated data and visual outputs serve as a foundation for further studies or optimizations in material and structural analysis.

Active ECM Remodeling and Void Closure

Additional images illustrating cell activity within the ECM, where cells align collagen fibers and fill gaps in the matrix, mimicking the pattern observed during the wound healing process.

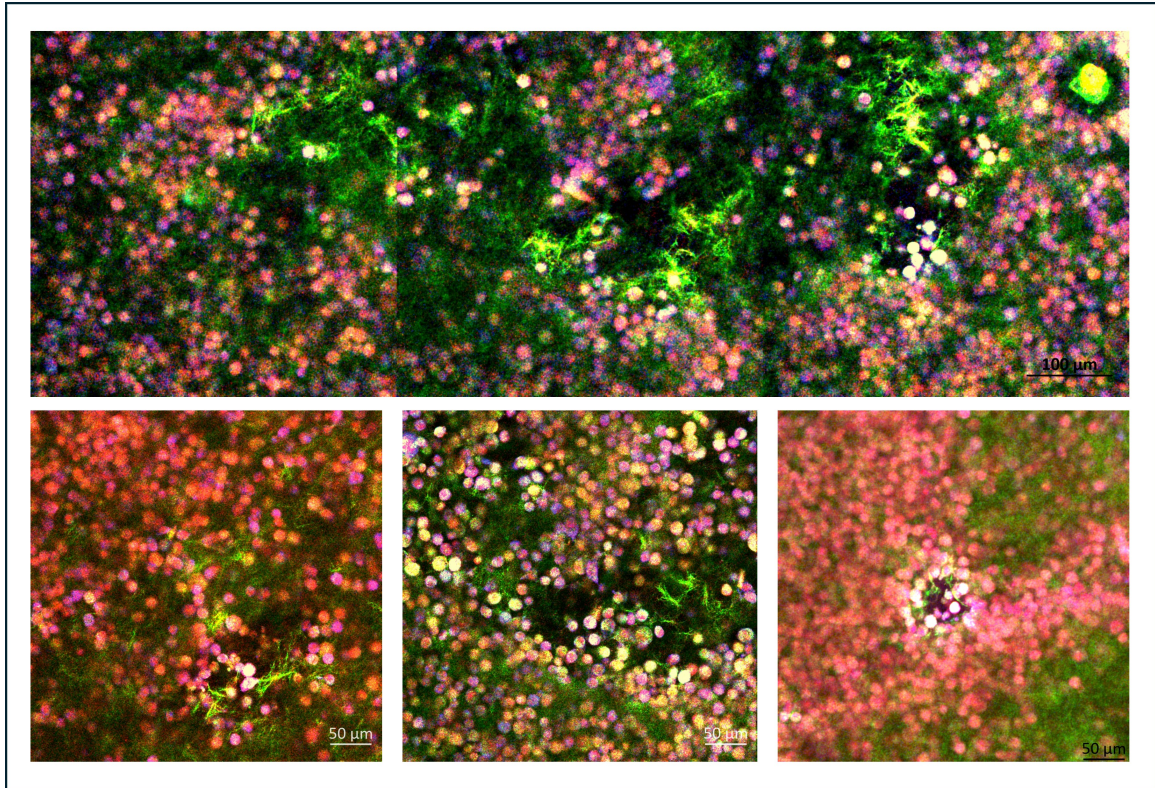


Figure A9: Active ECM Remodeling and Void Closure. *High-resolution fluorescence image showing a localized cavity within a densely populated cell region at different scales.*

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- [2] G. Stojkov, Z. Niyazov, F. Picchioni, R. K. Bose, Relationship between structure and rheology of hydrogels for various applications, *Gels* 7 (4) (2021) 255.