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# Synthesis and development of a phototunable MeHA-based 3D *in vitro* model of wound healing and fibrosis

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# Abstract

Wound healing is a tightly regulated process in which extracellular matrix composition, mechanics and immune-stromal interactions evolve over time. When this regulation is disrupted, pathological fibrosis can develop, leading to impaired tissue function. While conventional two-dimensional *in vitro* models have provided important mechanistic insight, they poorly recapitulate the three-dimensional, dynamic nature of tissue repair.

This thesis developed a mechanically tunable three-dimensional hydrogel-based *in vitro* platform based on methacrylated hyaluronic acid (MeHA), a biomaterial previously used in two-dimensional dynamic stiffening mechanobiology studies. To better support three-dimensional cell-matrix interactions and reduce confinement effects associated with dense hydrogel networks, MeHA was implemented as microgels assembling into a granular macroporous matrix and integrated into a microfluidic device compatible with standard imaging.

MeHA synthesis was optimized to reduce batch-to-batch variability and functionalization was quantified by  $^1\text{H}$  NMR. Hydrogels with tunable stiffness were formed via Michael-type addition and further stiffened *in situ* via secondary photo-crosslinking, enabling controlled stiffening to better reflect the evolving mechanics of wound healing and fibrosis. Rheological characterization confirmed that the platform spans stiffness ranges relevant to healthy and fibrotic dermal tissue and supports dynamic stiffening. Microgels were produced by mechanical fragmentation and co-loaded with primary dermal fibroblasts into microfluidic devices, forming a granular matrix compatible with immunofluorescence staining and fluorescence imaging. Finally, experimental methods were established for future immune cell integration: primary monocytes were isolated from human whole blood and purity was validated by flow cytometry for subsequent macrophage differentiation and incorporation.

Overall, this macroporous hydrogel system provides a reproducible platform to investigate how stiffness magnitude and mechanical history regulate cellular responses relevant to wound healing and fibrosis, with applicability beyond dermal models to other fibrotic tissues.

**Key-words:** wound healing; fibrosis; methacrylated hyaluronic acid; granular hydrogels; photo-stiffening; mechanotransduction



## Abstract in italiano

La guarigione delle ferite è un processo coordinato in cui composizione e proprietà meccaniche della matrice extracellulare (ECM), insieme alle interazioni tra cellule immunitarie e stromali, cambiano nel tempo. Quando la risoluzione fallisce, la riparazione può deviare verso la fibrosi, con perdita della funzione tissutale. Sebbene i modelli *in vitro* bidimensionali abbiano chiarito meccanismi importanti, non riproducono adeguatamente la natura tridimensionale e dinamica della riparazione tissutale.

In questa tesi è stata sviluppata una piattaforma tridimensionale a base di idrogel, con proprietà meccaniche modulabili, basata su acido ialuronico metacrilato (MeHA), già impiegato in studi bidimensionali di meccanobiologia con irrigidimento dinamico. Per favorire interazioni cellula-matrice più rappresentative e ridurre il confinamento tipico di reti idrogel dense, il MeHA è stato realizzato come microgel assemblati in una matrice granulare macroporosa e integrati in un dispositivo microfluidico compatibile con imaging.

La sintesi del MeHA è stata ottimizzata per ridurre la variabilità tra lotti e il grado di funzionalizzazione è stato quantificato tramite  $^1\text{H}$  NMR. Gli idrogel sono stati formati mediante addizione di tipo Michael e irrigiditi *in situ* tramite fotoreticolazione, ottenendo un incremento controllato della rigidità nel tempo. La caratterizzazione reologica ha confermato che la piattaforma copre intervalli di rigidità rilevanti per il derma sano e fibrotico. I microgel sono stati prodotti per frammentazione meccanica e co-caricati con fibroblasti dermici primari in dispositivi microfluidici compatibili con immunofluorescenza e imaging in fluorescenza. Infine, sono state poste le basi per l'integrazione della componente immunitaria: monociti primari sono stati isolati da sangue umano e la purezza è stata verificata mediante citofluorimetria per la successiva differenziazione in macrofagi da inserire nel sistema.

Nel complesso, la piattaforma proposta è riproducibile e adatta a studiare come l'intensità ed evoluzione temporale della rigidità regolino le risposte cellulari rilevanti per guarigione e fibrosi, con potenziale estensione oltre al modello dermico.

**Parole chiave:** guarigione delle ferite; fibrosi; acido ialuronico metacrilato; idrogeli granulari; irrigidimento mediante fotocrosslinking; meccanotrasduzione



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# 1 Introduction

When tissues are injured as a result of trauma, surgery, burns or disease, the body undergoes a highly coordinated biological process known as wound healing [1]. This process aims to rapidly restore tissue integrity and preserve barrier function through the coordinated action of immune cells, stromal cells and extracellular matrix (ECM) components. Understanding how this process unfolds, and also how it can fail, is fundamental to unravel the origins of pathological tissue remodeling, including fibrosis [2].

When repair becomes dysregulated, fibrosis can emerge as a common endpoint across multiple organs and tissue microenvironments. In idiopathic pulmonary fibrosis, repeated epithelial injury and dysregulated repair responses in the lung are associated with progressive ECM accumulation and stiffening that compromise gas exchange [3]. Following myocardial infarction, ischemia-driven cardiomyocyte death triggers the generation of fibrotic scar tissue that replaces functional myocardium, impairing contractility [4]. In chronic liver disease, sustained hepatocyte injury and inflammation promote activation of fibrogenic pathways, leading to excessive collagen deposition, architectural remodeling and loss of organ function [5].

Dysfunctional healing through fibrosis often causes lifelong disability and has a major impact on mortality and healthcare systems. It has been estimated that more than 40% of deaths worldwide are associated with fibrotic disorders, with approximately 45% of deaths in the United States linked to these disease processes [6]. In addition to its clinical burden, the economic cost of fibrotic diseases is significant and is estimated to be on the order of tens of billions of dollars [2].

In this thesis, the focus is placed on how ECM mechanics, particularly stiffness and its temporal evolution, regulate wound healing and fibrotic outcomes by shaping the behavior of key cellular players, namely fibroblasts and macrophages. Dermal wound healing is used as the reference case because skin mechanics and fibrosis are well characterized and human dermal fibroblasts are readily accessible, while the mechanobiological framework is relevant across fibrotic tissues.

The following sections first summarize the main phases of wound healing and how dysregulation can lead to fibrosis; they then introduce core mechanotransduction principles and review *in vitro* modelling strategies. Together, this motivates the development of a three-dimensional, mechanically tunable hydrogel platform that couples dynamic control of stiffness with a macroporous architecture to reduce

constraints on cell spreading, force generation and matrix remodeling compared to dense microporous hydrogel networks.

## 1.1. Wound healing

The ability to repair and replace damaged soft tissue is critical to the body's ability to respond to injury. While key differences in wound healing exist between different anatomical sites, stages of development and species, the fundamental steps (*e.g.* signaling pathways, cell populations involved) of the typical adult wound healing process are conserved among mammals and are shared between different organ systems [7].

Wound repair is a tightly regulated sequence of overlapping events that depends on coordinated interactions among multiple cell types, molecular signaling pathways and the extracellular matrix (ECM). When this coordination fails, healing can become dysfunctional, resulting in chronic wounds or progression toward pathological fibrosis. Fibrosis is characterized by persistent inflammation, excessive extracellular matrix deposition and myofibroblast accumulation; fibrotic tissues exhibit increased stiffness and reduced extensibility and they often fail to recover the functional properties of the original tissue [8]. Adult repair therefore typically ends with the formation of *ex novo* connective tissue rich in myofibroblasts and collagen fibers [8].

This complex process is generally divided into four overlapping phases that occur over different timescales, from hours after injury to months, ultimately leading to functional recovery (Figure 1.1).

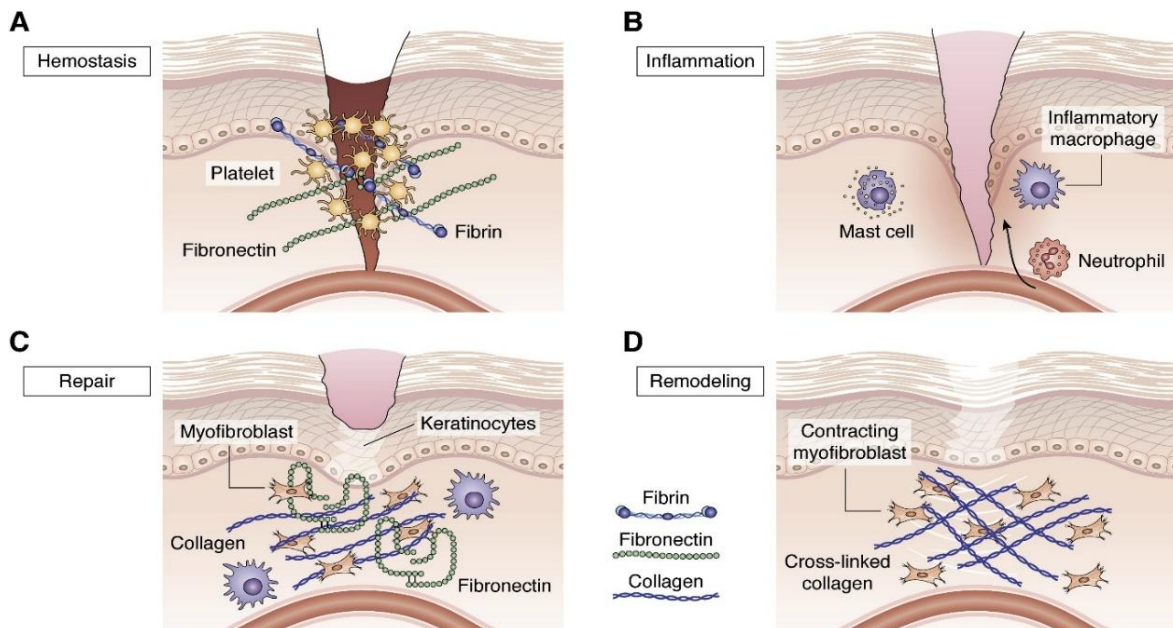


Figure 1.1: Phases of wound healing. Schematic overview of the main stages of tissue repair: A) Hemostasis with provisional matrix formation, B) Inflammation with immune-cell recruitment, C) Repair with provisional matrix maturation and ECM deposition, D) Remodeling with myofibroblast contraction and collagen crosslinking [8].

### 1.1.1. Hemostasis

Hemostasis starts immediately after injury and is responsible for stopping bleeding at the localized site of injury while maintaining a normal blood flow in the rest of the body [9].

Damage to resident cells and to the surrounding ECM triggers the coagulation cascade which leads to clot formation. At the same time, during this early phase, circulating platelets adhere to the damaged extracellular matrix and become activated, forming a provisional structure known as Early Provisional Matrix. This matrix is a fibrin-rich polymer containing crosslinked plasma fibronectin and serves as a temporary but stable scaffold that supports cell migration and invasion [10].

Platelets, anucleate cell fragments originating from megakaryocytes in the bone marrow and known as the first responders to injury, possess three organelles:  $\alpha$ -granules, dense granules and lysosomes [11]. Upon adhesion to damaged ECM,  $\alpha$ -granules are released from the platelets and deliver cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) which contribute to the initiation and regulation of the inflammatory response [12]. In addition, platelet activation during clot formation leads to local thrombin generation. Beyond its central role in converting fibrinogen into fibrin, thrombin also plays important signaling functions by promoting macrophage and neutrophil recruitment and by activating endothelial cells, thereby supporting angiogenesis through growth factor release [13], [14].

In parallel, tissue-resident macrophages contribute to the initiation of a local inflammatory response. These immune cells are originated from bone marrow progenitors that later migrated and matured within peripheral tissues, where they remain for the duration of their lifetime [8]. They can be activated by damage-associated signals released by injured or necrotic cells, including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) released following calcium fluxes in damaged epithelial and stromal cells, and danger-associated molecular patterns (DAMPs) such as high-mobility group Box 1 protein (HMGB-1) and extracellular ATP. These signals act as chemotactic cues and amplifiers of the pro-inflammatory cascade [15], [16].

The formation of a stable clot and the initiation of local inflammatory signaling marks the transition to the second phase of wound healing, inflammation.

### 1.1.2. Inflammation

The inflammatory phase of wound healing is characterized by early local signaling followed by recruitment and infiltration of circulating immune cells. This phase can be viewed as comprising an initial local inflammatory response, a rapid neutrophil response and a later stage dominated by monocyte infiltration and differentiation into macrophages. Importantly, the polarization of this cell type is fundamental for timely resolution of inflammation and progression towards repair [8].

Activated platelets play a central role in this phase by coordinating leukocyte recruitment through soluble mediators and cell-cell interactions. Upon activation, platelets translocate P-selectin from internal granules to their surface, enabling binding to P-selectin glycoprotein ligand-1 (PSGL-1) expressed on neutrophils, monocytes and eosinophils. In parallel, platelets release a range of chemokines and growth factors and also contribute to the local enrichment of mediators such as CXCL12 (stromal-derived growth factor 1, SDF-1) and hepatocyte growth factor (HGF), which regulate recruitment, proliferation and activation of multiple cell types involved in wound healing such as fibroblasts, neutrophils, monocytes, smooth muscle cells and mesenchymal stem cells [17], [18], [19].

At the same time, tissue-resident macrophages produce large amounts of proinflammatory cytokines (IL-1 $\beta$ , TNF, IL-6) in order to mobilize surrounding cells to respond, including neutrophils [20]. At this stage neutrophils cooperate with platelets in the clearance of debris and pathogens and also release pro-resolving soluble factors that polarize macrophages towards a repair phenotype [21].

Upon activation, platelets play a lot of different roles and interact with a lot of different immune cell types at this stage of wound healing (Figure 1.2).

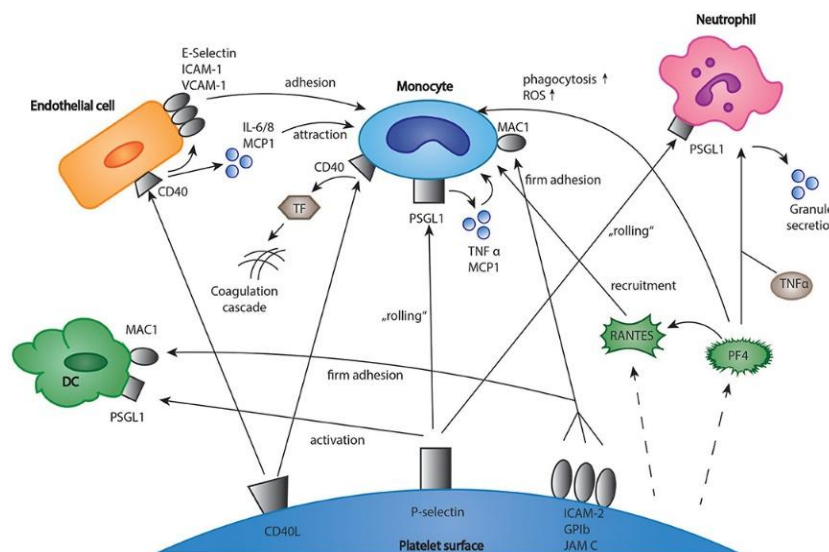


Figure 1.2: Platelet-immune cells interactions in the regulation of inflammation. Schematic overview of how activated platelets express membrane receptors and produce soluble mediators such as chemokines which regulate the inflammatory response of immune cells [18].

Although neutrophils are traditionally considered the first leukocyte population to infiltrate injured tissue, recent intravital multiphoton imaging studies performed on mice have demonstrated that neutrophil and monocyte recruitment can occur contemporaneously rather than in a strictly sequential manner [18], [19]. Monocyte infiltration occurs through sites of vascular leakage and involves distinct

subpopulations. Classical CD14<sup>+</sup> monocytes and non-classical CD16<sup>+</sup> monocytes have been described, with functional relevance toward angiogenesis and toward wound closure and ECM deposition, respectively [24], [25].

Following infiltration, monocytes differentiate into macrophages in response to tissue-specific biochemical signals released by platelets and neutrophils, as well as mechanical cues present within the wound environment. The polarization state adopted by these macrophages, toward pro-inflammatory or anti-inflammatory phenotypes, is fundamental for determining whether inflammation resolves appropriately or progresses toward chronic inflammation and fibrosis. It is important to take into consideration that patterns of leukocyte recruitment and behavior vary between organs due to differences in tissue architecture and physiology. Under physiological conditions, immune cell recruitment and inflammatory cytokine expression are spatially and temporally restricted; on the other hand, fibrotic disorders across tissues are characterized by sustained inflammatory activation of both tissue-resident and recruited immune cells [8].

### 1.1.3. Repair

The Early Provisional Matrix formed during the hemostasis undergoes progressive maturation as fibrin is gradually replaced by a fibronectin-rich transitional matrix that supports cell adhesion and migration and acts as a template for subsequent collagen assembly (Figure 1.3). Fibronectin fibrillogenesis is a key step in this transition because it provides an organizational scaffold that promotes collagen deposition and stabilization, enabling formation of a mechanically robust ECM. This transition from a fibrin-based scaffold to a fibronectin-rich matrix is driven primarily by inflammatory cells and fibroblasts through cell-mediated remodeling processes [9], [26].

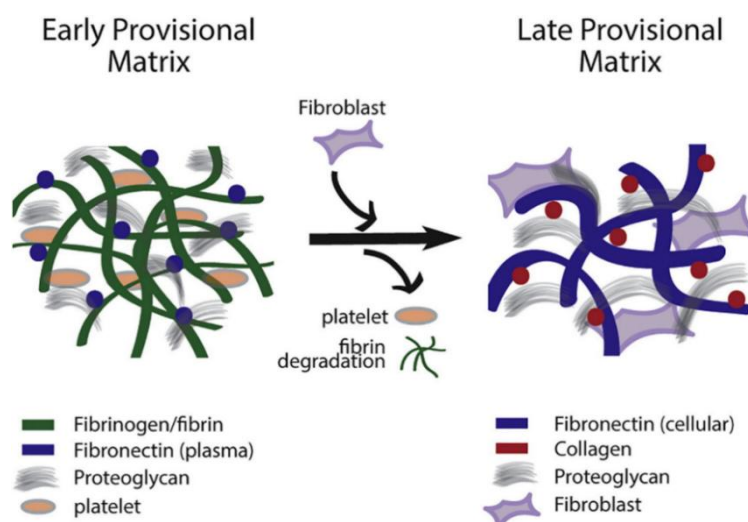


Figure 1.3: Schematic of the transition from early to late provisional matrix during wound repair. The early fibrin-rich matrix (with plasma fibronectin and platelets) is progressively remodeled by cells (primarily inflammatory cells and fibroblasts), leading to a late provisional matrix enriched in cellular fibronectin, collagen and proteoglycans [10].

Fibroblasts are key players during this phase. They migrate into the wound site and initially secrete fibronectin to support matrix maturation. In response to transforming growth factor- $\beta$  (TGF- $\beta$ ), largely secreted by macrophages and platelets, fibroblasts become activated and differentiate into myofibroblasts, a contractile cell type characterized by expression of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and increased collagen production. Myofibroblasts generate contractile forces and deposit collagen-rich ECM that restores structural integrity; however, excessive or persistent collagen deposition and crosslinking leads to pathological scarring and fibrosis [8], [27].

In skin wounds, keratinocytes migrate over the newly formed matrix and secrete vascular endothelial growth factor (VEGF), promoting angiogenesis and neovascularization to support tissue regeneration. Bidirectional crosstalk between keratinocytes and fibroblasts, mediated by growth factors and cytokines, plays a critical role in regulating the balance between regeneration and fibrosis during repair [28].

By the end of this phase, granulation tissue rich in myofibroblasts, macrophages, capillaries and collagen fibrils has formed.

#### 1.1.4. Tissue remodeling

The final phase of wound healing can persist for weeks to months and typically begins once myofibroblasts begin the contraction of the wound bed. During this process, myofibroblasts increase the expression of actin stress fibers and integrins to generate the forces required to realign the deposited ECM.

As the remodeling progresses, endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit the wound site leaving a tissue structure that contains few cells and mainly composed of collagen and ECM proteins. Macrophages play a central role by ingesting cell debris and contributing to the degradation of excess ECM deposited around the wound site [2]. After a few months, matrix metalloproteinases (MMPs) secreted by fibroblasts, macrophages and endothelial cells actively remodel the matrix, replacing the initially deposited type III collagen with a stronger type I collagen, thereby increasing the mechanical stability of the repaired tissue [29].

#### 1.1.5. Fibrosis: a result of dysregulated wound healing

Resolution of healing requires attenuation of inflammation, apoptosis or clearance of myofibroblasts and restoration of tissue homeostasis. When these regulatory mechanisms fail, wound healing transitions into a pathological state characterized by chronic inflammation and excessive extracellular matrix deposition, ultimately resulting in fibrosis [30].

At the molecular level, fibrotic progression is driven by persistent activation of pathways that are normally transient during physiological wound repair. Prolonged disturbance of the coagulation cascade, sustained TGF- $\beta$  platelet-derived growth

factor (PDGF) signaling and continue exposure to pro-inflammatory cytokines such as  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  promotes continuous fibroblast activation and myofibroblast differentiation. In parallel, unresolved inflammatory cues maintain macrophages in a pro-fibrotic activation state, reinforcing ECM deposition and tissue stiffening [31], [32].

Excessive accumulation of ECM components, particularly collagen, progressively alters the tissue mechanical properties by increasing stiffness. This mechanical remodeling is not merely a consequence of fibrosis, but actively regulates cellular behavior: increased matrix stiffness enhances integrin-mediated mechanotransduction in fibroblasts and macrophages, sustaining myofibroblast activation and inhibiting apoptosis. As a result, a positive feedback loop between inflammation, fibroblast activation and ECM stiffening is established, driving the persistence and progression of the fibrotic pathological microenvironment [31], [32].

### 1.1.6. Wound healing timescale

As introduced in the previous paragraphs, wound healing is not only defined by a sequence of biological events but also by distinct temporal dynamics that govern each overlapping phase. Although the exact duration of each phase can vary depending on the type of injury, tissue context and host conditions, several studies have identified characteristic time frames that describe the progression of the healing response [7], [33].

A representative overview of these temporal dynamics is provided in Figure 1.4 from desJardins-Park *et al* [7]. The x-axis reports time post-injury, while the y-axis qualitatively illustrates the relative predominance of each biological process over time. Importantly, the overlapping profiles emphasize that wound healing phases are not discrete or sequential events, but rather concurrent and dynamically evolving processes.

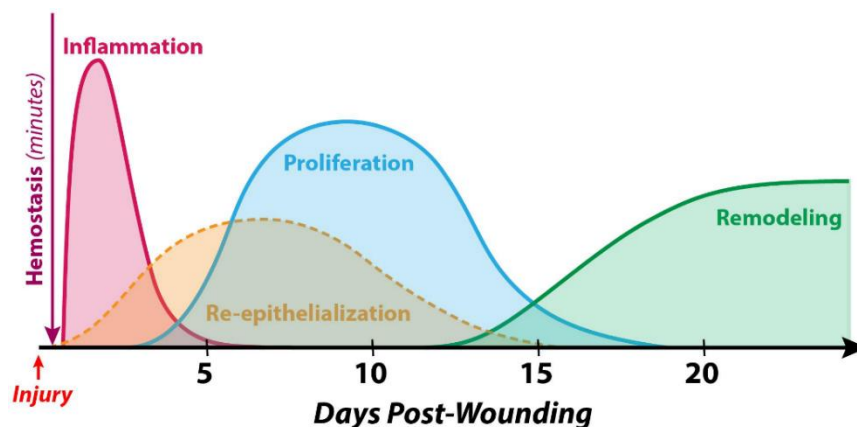


Figure 1.4: Timeline of normal human wound healing. Overlapping phases after injury (hemostasis, inflammation, proliferation and remodeling) shown as qualitative activity profiles over days post-wounding. The processes labelled as proliferation and re-epithelialization correspond to what in the thesis is described as the repair phase [7].

It is evident that hemostasis occurs immediately after injury and is rapidly followed by an inflammatory response, which then overlaps with subsequent reparative and remodeling processes. A physiological coordination and timely resolution of these phases are critical for functional tissue restoration. On the contrary, prolonged or dysregulated inflammation and repair have been associated with pathological outcomes, including chronic wounds and fibrotic tissue formation [31], [32].

These observations have important implications for *in vitro* modelling of wound healing and fibrosis. Reproducing not only the molecular composition but also the temporal evolution of the wound microenvironment, including time-dependent changes in mechanical properties, is essential to capture differences in cell behavior and phenotype [34].

## 1.2. How do cells sense the environment?

*In vivo*, cells reside within complex and dynamic physical environments and continuously experience mechanical cues that arise from tissue architecture, cell-generated forces and ECM organization. Cellular mechanotransduction refers to the processes by which cells sense these mechanical stimuli and convert them into biochemical signals that regulate gene expression and cell behavior [35].

Mechanical inputs relevant to physiological and pathological contexts include hydrostatic pressure, tensile and compressive forces, fluid shear stress, ECM stiffness or elasticity and changes in extracellular fluid viscosity. These mechanical cues are not confined to specific organs, but are often studied in settings where they are particularly prominent or clinically relevant. For example, fluid shear stress and hydrostatic pressure are investigated in blood vessels; tensile force, hydrostatic pressure and fluid shear force are central in the urinary bladder; fluid shear force and tensile force are relevant in the intestine; changes in ECM stiffness are a characteristic feature of fibrotic tissues, including liver and skin (Figure 1.5). These cues play essential roles in tissue development, homeostasis, repair and disease progression, including wound healing and fibrosis [35], [36], [37].

Cells detect mechanical cues primarily through specialized mechanosensitive structures located at the plasma membrane, including adhesion receptors (*e.g.* integrins that bind ECM ligands, often via RGD motifs), mechanically gated ion channels and growth factor receptors. These sensors are mechanically coupled to the cytoskeleton, enabling rapid transmission of forces across the cell. In contrast to biochemical signaling, which often relies on diffusion-limited processes, mechanical signals can propagate almost instantaneously through cytoskeletal networks. As a consequence, mechanotransduction provides cells with a fast and spatially integrated mechanism to probe their physical surroundings and adjust their behavior

accordingly. Mechanical inputs sensed at the cell surface are transmitted to intracellular signaling hubs and ultimately to the nucleus, where they influence chromatin organization and transcriptional activity, as discussed in Section 1.2.1 [37].

The concept of tensegrity, originally introduced as an architectural principle and later adapted to cell biology, is useful for understanding cellular mechanotransduction. In this model, cells are viewed as prestressed structures in which mechanical forces are distributed across interconnected elements, including the ECM, adhesion complexes, cytoskeleton and nuclear scaffold. Perturbations in extracellular mechanics can therefore propagate through this network and lead to structural and biochemical responses throughout the cell [36], [38]. In practice, changes in ECM stiffness alter the balance between cytoskeletal tension and resistance at adhesion sites, thereby regulating focal adhesion maturation, actomyosin contractility and force transmission to the nucleus.

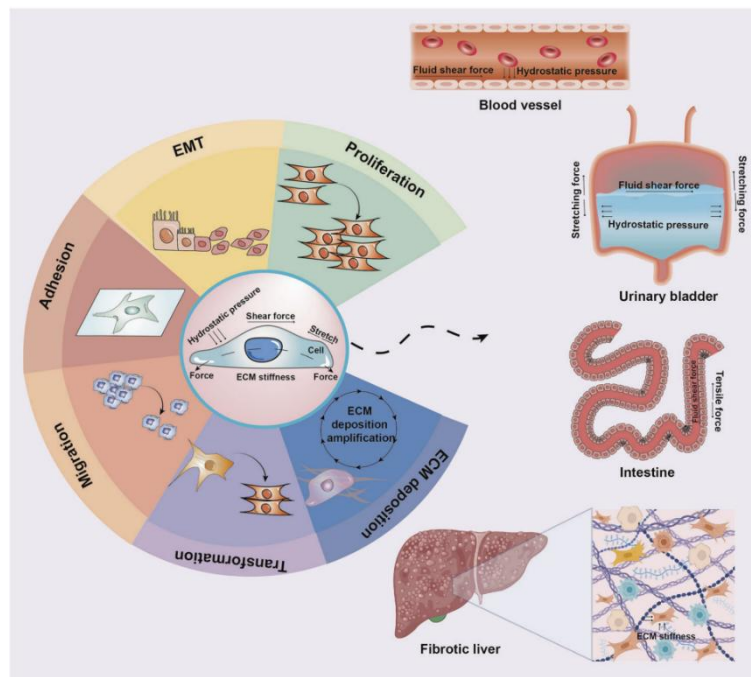


Figure 1.5: Cellular mechanotransduction in tissues and organs. Examples of prominent mechanical cues across tissues (shear stress, hydrostatic pressure, tensile forces, and ECM stiffness, including fibrotic stiffening) in physiological and pathological contexts [35].

### 1.2.1. ECM stiffness

Among the various mechanical signals to which cells are exposed to, ECM stiffness has emerged as a particularly important regulator of cell behavior. Tissue stiffness varies widely across organs and changes dynamically during development, wound healing and disease. In healthy tissues, ECM elasticity is tightly regulated to support normal cellular functions. In contrast, pathological processes such as fibrosis are characterized by excessive ECM deposition and crosslinking, leading to progressive tissue stiffening. Importantly, changes in ECM stiffness are not merely passive consequences of disease

but actively shape cellular responses through mechanotransduction pathways [36], [39].

Cells sense ECM stiffness primarily through integrin-mediated cell-ECM adhesions, which mechanically couple the extracellular matrix to the intracellular actin cytoskeleton. Integrins are heterodimeric transmembrane receptors composed of  $\alpha$  and  $\beta$  subunits, with extracellular domains that bind ECM ligands and cytoplasmic tails that associate with cytoskeletal and signaling proteins. Upon engagement, integrins cluster into multiprotein assemblies known as integrin adhesion complexes which include talin, vinculin, paxillin, focal adhesion kinase (FAK) and other adaptor proteins. These structures function both as mechanical anchoring sites and as signaling platforms that integrate biochemical and mechanical information from the microenvironment [40], [41].

Integrin-based adhesion play a central role in creating a bidirectional mechanotransduction, enabling both “outside-in” transmission of environmental mechanical cues to the cell and “inside-out” transmission of intracellular traction forces to the ECM [35], [42].

The organization, maturation and lifetime of integrin-mediated adhesions are highly sensitive to matrix stiffness. On compliant substrates, adhesions typically remain small and transient, while increasing ECM stiffness promotes adhesion growth, reinforcement and stronger coupling to actomyosin contractility. This stiffness-dependent maturation enhances intracellular tension and activates downstream signaling pathways, including RhoA/ROCK and FAK-mediated cascades ultimately regulating cytoskeletal organization, cell shape, migration and differentiation [40], [43].

Mechanical signals transmitted through focal adhesions propagate along actin filaments toward the nucleus. The cytoskeleton is mechanically connected to the nucleus via linker complexes, allowing forces to directly influence nuclear shape and mechanics. Nuclear mechanosensing affects chromatin organization and accessibility, therefore modulating transcriptional responses. In this context, transcriptional co-activators such as Yes-associated protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ) have emerged as central mediators of stiffness-dependent gene regulation. In fact, increased matrix stiffness promotes nuclear localization of YAP/TAZ and activation of transcriptional programs associated with cell proliferation, ECM synthesis and survival [36], [44].

In the context of wound healing, ECM stiffness is not static but evolves over time as provisional matrix is deposited, remodeled and, in some cases, excessively crosslinked. This temporal change in stiffness provides a mechanical context that can promote fibroblast activation and influence macrophage phenotype, thereby affecting whether repair resolves or progresses toward persistent scarring and fibrosis [45]. For

this reason, modulating and probing ECM stiffness is central to mechanistic *in vitro* models of wound healing.

### 1.2.2. Fibroblasts-Myofibroblasts activation

Fibroblasts are a heterogeneous population of tissue-resident mesenchymal cells that maintain ECM homeostasis but rapidly change phenotype after injury to support repair. In the presence of danger signals, such as ECM disruption or inflammation, fibroblasts suspend their homeostatic roles and start a process called fibroblast-myofibroblast activation which is characterized by increased migration, proliferation and secretion of fibrous, collagen-rich ECM [46], [47].

Transient activation into contractile myofibroblasts is beneficial for closing wounds; however, persistent activation drives organ scarring and fibrosis, making the temporal control of this switch a decisive factor for a desired outcome [48]. Functionally, myofibroblasts are defined by their ability to exert contractile force through actomyosin bundles (stress fibers); cells that lack these contractile structures should not be classified as myofibroblasts even if they express specific marker proteins [49].

Fibroblast-myofibroblast activation is a staged process (Figure 1.6): (proto-)myofibroblasts form nascent actin bundles and, with appropriate signals, mature into  $\alpha$ -SMA incorporating highly contractile myofibroblasts; incorporation of  $\alpha$ -SMA into pre-existing actin filaments increases contractile output which is a mechanistic key aspect of the mature myofibroblast [50].

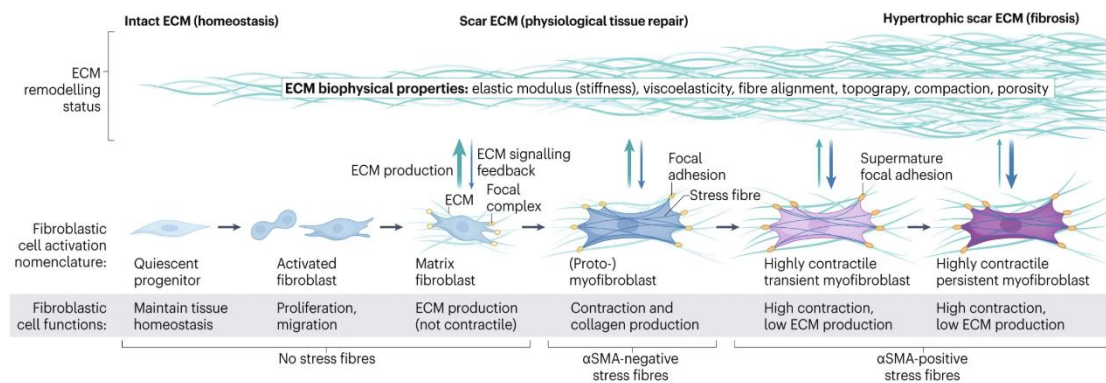


Figure 1.6: Mechanically driven progression of fibroblast activation during repair and fibrosis. Schematic linking ECM remodeling (from intact ECM to scar ECM and hypertrophic scar) to a stepwise increase in fibroblast contractility: quiescent/activated fibroblasts progress to (proto-)myofibroblasts as increasing ECM resistance promotes stress-fiber formation,  $\alpha$ -SMA incorporation and maturation of cell-ECM adhesions from focal complexes to supermature focal adhesions. The model highlights a positive mechanical feedback loop in which myofibroblast contraction further stiffens the ECM, stabilizing the contractile phenotype, and distinguishes transient myofibroblasts in physiological repair from persistent myofibroblasts in fibrosis. Adapted from [46].

Importantly, biochemical cues such as TGF- $\beta$  are major drivers of fibroblast activation and often promote the acquisition of myofibroblast-associated features; however, they are not universally required for myofibroblast-like phenotypic switching [51], [52]. Instead, stable emergence and persistence of contractile features (*e.g.* robust stress fibers and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) incorporation) depend on whether the surrounding matrix provides sufficient mechanical resistance to sustain force transmission. ECM mechanics therefore acts as a key checkpoint that, together with soluble cues, determines whether fibroblasts resolve toward a homeostatic state or progress toward persistent activation [27], [36].

Moreover, cell-generated forces mechanically activate latent TGF- $\beta$  stored in the ECM, while active TGF- $\beta$  enhances cellular contractility and ECM production, creating a feed-forward loop that can lock tissues into a profibrotic state [53], [54]. Fibroblasts actively remodel their matrix promoting fibronectin fibrillogenesis, collagen deposition, fiber alignment and enzymatic crosslinking. These processes progressively increase tissue stiffness and alter the ECM microenvironment; in turn, these matrix changes amplify integrin-mediated force transmission and reinforce myofibroblast activation [46], [55].

Despite this framework, it remains challenging to disentangle the contributions of biochemical and mechanical cues in physiologically relevant three-dimensional environments, because many mechanistic insights were established in two-dimensional culture, where cells spread readily and are not constrained by matrix microarchitecture. In three-dimensional hydrogels, spreading and sustained tension can be limited by nanoporous mesh size and by whether cells must proteolytically remodel the network to create space. As a consequence, stiffness-dependent phenotypes observed in two dimensions may not translate directly to three-dimensional matrices, because confinement and remodeling requirements can become dominant regulators of cell shape, traction and downstream activation outputs [56].

In parallel with *in vitro* studies, biomechanical and modelling studies have characterized thresholds of substrate stiffness and traction that allow stabilization of stress fibers and focal adhesions. When cellular traction exceeds these thresholds, the contractile phenotype is mechanically reinforced and myofibroblast persistence is favored, whereas below the threshold mechanical environments permit phenotype reversal to fibroblasts [49].

### 1.2.3. Macrophages

Macrophages, historically described as professional phagocytes clearing cellular debris and pathogens, are now recognized as key orchestrators of ECM remodeling and fibroblast activation throughout wound healing. Their ability to adopt both pro-

and anti-fibrotic roles, often mediated by distinct macrophage subpopulations, places them at the interface between inflammation resolution and pathological fibrosis [57].

During wound healing, macrophages undergo dynamic phenotypic transitions in response to environmental cues: early after injury they support clearance and initiation of repair, whereas later stages promote tissue remodeling and inflammation resolution [58]. Macrophage activation is increasingly understood as a continuum rather than discrete subsets, with phenotypes adapting according to microenvironmental signals [59], [60].

A defining macrophage function in wound healing is the regulation of fibrogenesis through soluble mediators. They secrete profibrotic cytokines such as TGF- $\beta$ 1 and PDGF, which directly stimulate fibroblast activation, proliferation, migration and differentiation into myofibroblasts [61]. In parallel, they regulate ECM turnover by balancing MMPs and their inhibitors, thereby controlling both matrix deposition and degradation. Macrophage-derived chemokines recruit fibroblasts and inflammatory cells, amplifying fibrotic responses indirectly [57].

Phagocytosis represents another critical macrophage function linking inflammation and fibrosis. Clearance of apoptotic versus necrotic cells defines distinct macrophage responses: phagocytosis of apoptotic cells typically represses inflammation, whereas removal of necrotic cells promotes inflammatory signaling [62]. Since phagocytosis of dying cells increases TGF- $\beta$ 1 secretion, macrophage engulfment activity can be profibrotic; conversely, clearance of apoptotic myofibroblasts and cellular debris can contribute to fibrosis resolution by removing sources of profibrotic signaling [57], [63].

Beyond biochemical signaling, macrophages are highly responsive to mechanical cues within the ECM. Substrate rigidity and tissue architecture influence macrophage activation and polarization via cell-matrix interactions (including integrins) and mechanosensitive ion channels [64]. Among these, the mechanosensitive ion channel Piezo1 has emerged as a key regulator of macrophage mechanobiology [65]. Piezo1-mediated  $\text{Ca}^{2+}$  influx is activated by increased membrane tension induced by mechanical stress or elevated substrate stiffness [66]. Macrophages lacking Piezo1 display reduced expression of pro-inflammatory markers and increased expression of pro-healing markers, suggesting that Piezo1 signaling in stiff environments can bias macrophage toward inflammatory phenotype [67].

In addition to stiffness, ECM architecture can influence macrophage behavior during wound healing. Migration through fibrous matrices imposes geometric and cytoskeletal constraints that alter cell shape and organization [68] and macrophage elongation in fibrillar environments has been proposed as a mechanical cue contributing to polarization [69].

However, much of our mechanistic understanding of macrophage mechanosensing comes from simplified in vitro settings (often two-dimensional) and frequently from murine or immortalized models, which may not fully recapitulate primary human

macrophage behavior in three-dimensional tissue-like ECM. As a result, linking defined mechanical and architectural features of the ECM to macrophage state in physiologically relevant 3D contexts remains an active area of investigation [67], [68], [69].

### 1.2.3.1. Fibroblast-Macrophage crosstalk

During tissue repair and fibrosis, macrophages and fibroblasts engage in a tightly regulated bidirectional crosstalk that integrates biochemical signaling with ECM remodeling. *In vivo* studies across multiple organs demonstrate that reciprocal signaling between these two cell types governs the balance between regenerative repair and dysfunctional wound healing resulting in persistent fibrosis (Figure 1.7) [59].

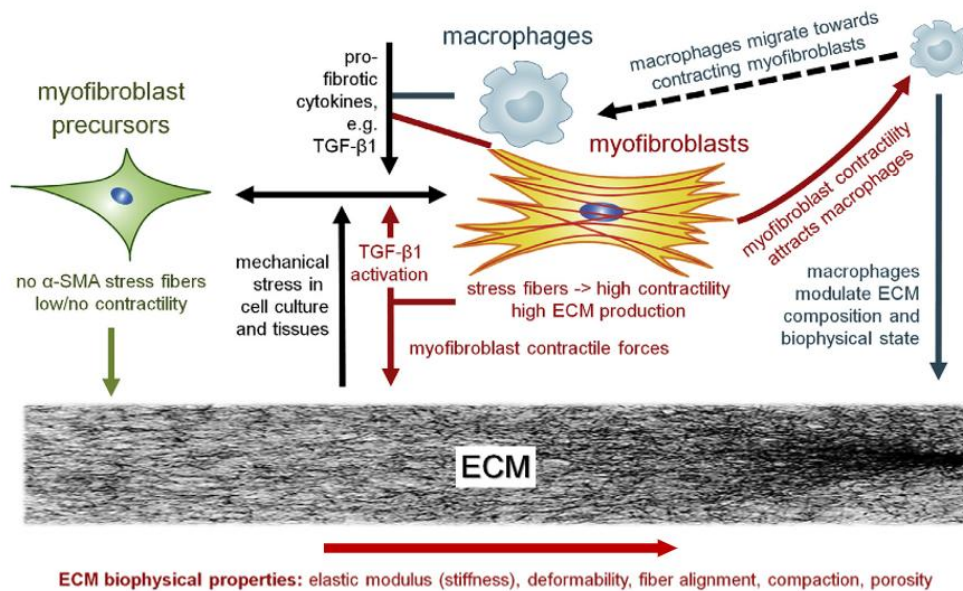


Figure 1.7: Schematic overview of macrophage-myofibroblast-ECM crosstalk in repair and fibrosis. Profibrotic cytokines (e.g. TGF- $\beta$ 1), together with mechanical stress transmitted through the ECM, promote activation of myofibroblast precursors. Contractile ( $\alpha$ -SMA) myofibroblasts remodel and stiffen the ECM, which reinforces profibrotic signaling by enhancing mechanosensing and mechanical activation of latent TGF- $\beta$ 1 stored in the matrix, creating a positive feedback loop. The schematic also highlights macrophage recruitment toward contracting myofibroblasts and macrophage-derived contributions of additional TGF- $\beta$ 1 and profibrotic mediators within the remodeled microenvironment. Adapted from [56].

Biochemical cues reinforce this bidirectional communication. Activated fibroblasts secrete macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), promoting macrophage survival and

differentiation toward pro-fibrotic phenotypes [70]. Conversely, macrophages represent a major source of TGF- $\beta$  which drives fibroblast-to-myofibroblast differentiation, ECM deposition and sustained fibroblast activation [71]. Additional mediators, particularly PDGFs, enhance fibroblast migration and proliferation [59], [72].

The ECM itself acts as an active signaling platform in fibroblast-macrophage communication. Both cell types engage ECM components via integrins, specifically macrophage  $\alpha v \beta 3$  binding fibronectin and fibroblast  $\alpha v \beta 1$  or  $\alpha 5 \beta 1$  interacting with collagen, thereby coupling biochemical signaling to mechanotransduction [73]. In both macrophages and fibroblasts, FAK integrates ECM stiffness and tensile forces into intracellular signaling pathways that reinforce activation and matrix remodeling [74]. Moreover, the ECM serves as a reservoir for latent TGF- $\beta$ , which can be mechanically activated by cellular contraction, sustaining profibrotic signaling within stiffened microenvironments [59].

In addition to soluble mediators and ECM-bound signaling, mechanical cues have emerged as an additional layer regulating fibroblast-macrophages crosstalk. Myofibroblasts indirectly regulate macrophage behavior by remodeling the ECM, thereby generating mechanically stiff microenvironments. In fact, macrophages are highly mechanosensitive and able to sense changes in ECM stiffness; as a consequence, they modulate their spreading, morphology, migration, podosome formation, phagocytic capacity, cytoskeletal organization, proliferation and cytokine secretion, as it has been shown in both *in vitro* systems and *in vivo* fibrosis models [68], [75]. Consequently, ECM-induced alterations in macrophage shape and cytoskeletal tension further influence macrophage polarization and functionality [59], [69], [72].

Beyond indirect mechanical signaling, contracting myofibroblasts may also exert direct long-range mechanical guidance on macrophages through aligned fibrillar collagen networks. This phenomenon has been demonstrated in 3D collagen matrices, suggesting that fibroblast-generated forces can spatially organize immune cell migration within remodeling ECM [59].

### 1.3. *In vitro* models of wound healing and fibrosis

*In vitro* models have become indispensable tools to investigate the cellular and molecular mechanisms underlying wound healing and fibrosis. Although traditional two-dimensional (2D) cell cultures have historically provided fundamental insights into cellular functions and signaling pathways, their simplicity fails to capture the heterogeneity, spatial organization and dynamic characteristics of living tissues. As a

result, *in vivo* animal models have long been employed to study tissue repair and fibrotic disease, offering an integrated representation of cellular, biochemical and mechanical processes. However, the use of animal models comes with substantial limitations including ethical concerns, high costs, limited experimental throughput and difficulties in isolating specific microenvironmental variables [76], [77].

In response to these challenges, Russell and Burch introduced the 3R principle, Replacement, Reduction, and Refinement, in 1959, establishing a framework to minimize animal use in experimental research. This concept has since been reinforced by regulatory policies such as the European Directive 63/2010 which promotes the development and adoption of non-animal-based experimental approaches. Within this context, *in vitro* systems represent a valuable strategy to reduce reliance on animal models while enabling controlled and reproducible experimentation [76].

Cell culture techniques allow cells isolated from tissues to be propagated under tightly regulated conditions including temperature, pH, oxygen tension and growth factor availability, thereby reducing experimental variability and enhancing reproducibility. Despite these advantages, conventional 2D monolayer cultures remain a limited representation of the *in vivo* microenvironment. In conventional 2D monolayer culture on tissue-culture plastic, cell-cell interactions are largely planar, tissue-specific three-dimensional architecture is absent and interactions with a physiologically structured ECM are minimal or reduced to simple protein coatings. Because the ECM *in vivo* functions not only as a structural scaffold, but also as a reservoir of growth factors and bioactive molecules, these simplified culture conditions can alter key signaling pathways and cellular behaviors [77], [78].

To overcome these limitations, three-dimensional cell culture models have been developed over the past decades. Unlike 2D systems, 3D cultures enable cells to interact with their surroundings in all spatial dimensions, promoting more physiologically relevant cell-cell and cell-ECM interactions and better recapitulating tissue-like morphology and organization. In addition, 3D systems naturally generate gradients of nutrients, oxygen and soluble factors, conditions that more closely resemble native tissues and pathological environments such as fibrosis [77].

Importantly, while 3D models provide increased biological complexity, they are increasingly regarded as a conceptual and practical bridge between simplified 2D cultures and complex *in vivo* systems [37].

Within wound healing and fibrosis research, this hierarchy of models, from 2D monolayers to scaffold-based 3D cultures, organoids and microengineered systems, allows investigators to progressively increase biological complexity while maintaining experimental control. Among these approaches, hydrogel-based 3D models have gained particular relevance due to their high water content, tissue-like mechanics and

ability to mimic key features of the native ECM. Compared to conventional two-dimensional culture on tissue-culture plastic, three-dimensional hydrogel cultures enable cells to receive biochemical and mechanical signals in all spatial dimensions and allow independent control over matrix stiffness, composition and structure. These features make hydrogel systems particularly suitable for investigating mechanotransduction processes involved in wound healing and fibrosis, where dynamic changes in ECM mechanics and composition play a central regulatory role [79], [80].

### 1.3.1. 2D *in vitro* studies

Numerous studies have employed 2D monoculture models to examine macrophage or fibroblast responses to defined material properties [81], [82]. These approaches typically involve culturing a single cell type on substrates with controlled chemistry, topography or stiffness, enabling the isolation of specific biophysical cues. Additional complexity has been introduced to this simplified systems through conditioned media experiments or indirect co-culture models, in which macrophages and fibroblasts are separated by transwell inserts to study paracrine signaling without direct cell-cell contact [83].

A notable example of material-driven 2D modelling was reported using polymeric substrates with systematically varied surface properties including roughness, wettability and chemical functionality. In this system, macrophages or fibroblasts were cultured on different polymer rod models of polycaprolactone (PCL) or two copolymers, poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT), subjected to physical, chemical or plasma-based surface modifications. These studies showed that cell attachment and proliferation, pro-inflammatory cytokine (IL1 $\beta$  and IL6) and anti-inflammatory cytokine (TGF $\beta$ 1 and IL10) secretion and collagen synthesis are significantly modulated by material cues (*e.g.* micro- and nano-patterns) in 2D environments [83], [84].

To further interrogate fibroblast-macrophage crosstalk, self-assembled monolayer biomaterials with tunable surface charge and wettability were combined with compartmentalized culture systems to enable controlled co-culture of macrophages and fibroblasts. In this model, hydrophobic surfaces elicited a pronounced pro-inflammatory response, whereas hydrophilic and anionic surfaces reduced inflammatory signaling. Importantly, macrophage motility was enhanced in the presence of fibroblasts compared to monoculture conditions, highlighting the bidirectional nature of macrophage-fibroblast interactions even in simplified 2D systems [83], [85].

Beyond biochemical cues, 2D culture systems have played a fundamental role in elucidating how cells sense and respond to mechanical properties. It has been demonstrated that cells, including fibroblasts, actively probe substrate stiffness through adhesion complexes and the actomyosin cytoskeleton, translating mechanical inputs into changes in cell morphology, contractility and phenotype [86], [87]. These studies established substrate stiffness as a key regulator of cell fate and function, with profound implications for development, regeneration and fibrotic disease.

More recent 2D studies have leveraged dynamically tunable hydrogels to better mimic the evolving mechanical landscape of wound healing. Phototunable hydrogels in which stiffness can be increased *in situ*, in the presence of cells, were introduced in the past decades; in these systems, both short- and long-term cellular responses were showed to depend not only on absolute stiffness, but also on the timing of mechanical changes. In this platform, mesenchymal stem cells responded differently to early versus late stiffening, providing early evidence that cells retain a form of mechanical memory [88].

A similar dynamic stiffening strategy using methacrylated hyaluronic acid (MeHA) hydrogels was later applied for fibrotic modelling to study hepatic stellate cell activation. Although conducted in a 2D context, this work demonstrated that progressive matrix stiffening alone was sufficient to drive myofibroblast activation, as evidenced by enhanced cytoskeletal organization and mechanotransduction signaling [89].

Collectively, 2D *in vitro* approaches have been instrumental in defining core principles of wound healing and fibrosis, particularly the roles of substrate mechanics, material properties and fibroblast-macrophage crosstalk. However, their inability to reproduce three-dimensional force transmission, matrix remodeling and spatially heterogeneous mechanical cues has driven the development of 3D *in vitro* models designed to more faithfully recapitulate complex *in vivo* systems [77].

### 1.3.2. 3D *in vitro* studies

Three-dimensional *in vitro* models have emerged as a critical advancement for studying wound healing and fibrosis, addressing key limitations of two-dimensional cultures while promoting more physiologically relevant cell-microenvironment interactions [77], [90].

Among available 3D platforms, hydrogel-based systems have become especially prominent due to their high-water content, tissue-like mechanics and compatibility with a wide range of cell types. Hydrogels enable independent tuning of matrix stiffness, degradability and biochemical ligand presentation, allowing controlled presentation of ECM cues that dynamically evolve during wound healing and fibrosis.

Importantly, these systems naturally generate gradients of nutrients, oxygen and soluble factors, further enhancing physiological relevance [77], [79].

The relevance of 3D culture has been particularly evident in skin and connective tissue models where architecture and ECM organization are central. Early 3D skin equivalents generated using collagen-based matrices enabled improved stratification compared to 2D systems, while subsequent incorporation of fibroblasts enhanced dermal-epidermal interactions and tissue maturation. These models provided physiologically relevant platforms for studying wound healing while offering alternatives to animal experimentation [77].

Beyond structural tissue models, *in vitro* biomaterial systems (including two-dimensional screening approaches) have been used to dissect macrophage-fibroblast interactions during fibrosis and to show that experimental test conditions and scaffold design can strongly influence the observed fibrotic response, with co-culture studies highlighting how biomaterial context modulates macrophage-driven fibroblast recruitment [83], [91].

The influence of matrix mechanics in 3D environments has also been shown to diverge from previously established 2D paradigms. Using microengineered pulmonary interstitial mimetics, myofibroblast differentiation was found to correlate positively with matrix architecture and degradability rather than stiffness alone. More broadly, in nanoporous hydrogels (*e.g.* PEG-based systems), stiffness-dependent phenotypes can be masked or shifted by additional variables such as the extent of protease-mediated network remodeling to enable cell spreading; in this scenario, “stiffness” is not the only barrier, because cells may need to create space by degrading the matrix, and this degradative requirement can become a dominant driver of phenotype compared with stiffness itself [56].

Similarly, engineered 3D collagen networks with tunable stiffness and glycosaminoglycan functionalization revealed that macrophage polarization during wound healing is highly sensitive to matrix dimensionality, mechanics and biochemical composition. In these systems, macrophages exhibited polarization profiles opposite to those observed in 2D substrates, emphasizing that planar cultures may not correctly represent immune responses relevant to tissue repair and fibrosis [92]. However, collagen-based systems often intrinsically couple mechanics to matrix architecture and pore size, which complicates isolating purely mechanical effects on cellular phenotypes. In addition, collagen-rich matrices can limit downstream readouts of fibroblast-derived collagen deposition, a key functional hallmark of fibrosis, because the scaffold itself contains collagen background [91].

Collectively, these studies show that 3D *in vitro* models uncover mechanobiological behaviors of fibroblasts and macrophages that cannot be captured in 2D systems and that matrix microarchitecture and remodeling constraints are central determinants of cell phenotype in 3D. These considerations motivate three-dimensional platforms that

better decouple stiffness from confinement and remodeling requirements, while maintaining controlled biochemical presentation, an approach that supports the rationale for macroporous, tunable hydrogel architectures.

### 1.3.3. Granular hydrogels as macroporous biomaterials

Conventional bulk hydrogels are typically formed as dense polymer networks with nanoscale mesh sizes. While these materials provide an ECM-like hydrated environment, their pore architecture can be suboptimal by restricting nutrient transport and cell-cell interactions; moreover, crosslinking a bulk hydrogel around cells in suspension can entrap cells within a nanoscale mesh that limits growth, connectivity, tissue development and other basic physiological processes [93], [94].

To address these constraints, bulk hydrogels can be transformed into granular architectures by breaking them into microgel building blocks and assembling them into jammed scaffolds, thereby positioning cells at discontinuities and within interstitial void space between granules. Granular hydrogels are particularly attractive for *in vivo* delivery because microgel assemblies can fluidize and exhibit shear-thinning under applied stress, facilitating injection and localized placement. In addition, they can be used as macroporous scaffold systems that provide cells with a three-dimensional environment and reduced geometric confinement compared with dense bulk networks. In this configuration, granular hydrogels provide an interconnected porous network originating from the spaces between adjacent microgels, offering improved mass transport and more permissive space for cell migration and tissue formation relative to conventional nanoporous hydrogels [93], [94].

To produce microgels for granular hydrogel assembly, several fabrication strategies can be used. Microfluidic emulsions, lithography and electrohydrodynamic spraying offer high control over microgel size and shape but are often low-throughput and challenging to scale, whereas batch emulsions and mechanical fragmentation can rapidly produce large amounts of microgels with higher variability in particle geometry. Importantly, mechanical fragmentation can avoid the need for subsequent removal of emulsion-phase chemicals, simplifying use in biologically sensitive applications [95].

### 1.3.4. Methacrylated hyaluronic acid-based hydrogels

Among hydrogel-based 3D culture systems, hyaluronic acid (HA) derived materials have gained particular attention due to their intrinsic biological relevance in wound healing and fibrotic processes. HA is a non-sulphated glycosaminoglycan widely distributed in the ECM of tissues such as skin, cartilage and connective tissue, where it contributes to matrix organization, hydration and cell signaling. During tissue repair, HA plays a dynamic role in regulating inflammation, cell migration and matrix remodeling, while dysregulated HA turnover has been implicated in chronic

inflammation and fibrotic disease. These properties make HA an especially relevant ECM component to incorporate into *in vitro* models that aim at recapitulating wound healing and fibrosis [81], [96].

Beyond its biological relevance, HA offers significant advantages as a biomaterial due to its extensive chemical tunability. The HA backbone contains multiple functional groups that can be modified to enable a wide range of crosslinking strategies, allowing precise control over hydrogel structure, mechanics and degradation behavior. HA-based hydrogels can be fabricated through physical interactions, enzymatic reactions, Michael-type additions or radical polymerization, enabling the generation of matrices with tissue-relevant stiffness [79], [96].

However, a key limitation of native HA is its lack of intrinsic integrin-binding motifs, which can restrict cell adhesion and spreading in *in vitro* systems. While HA interacts with cell-surface receptors such as CD44, many cell types, including dermal fibroblasts, require the incorporation of adhesive proteins or peptides to support stable attachment and cytoskeletal organization. To resolve this limitation, HA is frequently combined with ECM proteins such as collagen or fibronectin or chemically functionalized to incorporate adhesive ligands (*e.g.* RGD peptide) [79], [81].

Methacrylated hyaluronic acid (MeHA) represents one of the most widely used HA derivatives for *in vitro* mechanobiology studies. MeHA is typically synthesized by reacting HA with methacrylic anhydride, introducing methacrylate groups that enable different crosslinking strategies. This chemistry allows hydrogels to be formed under cytocompatible conditions with spatial and temporal control, offering precise regulation over network density and mechanical properties: by tuning parameters such as HA molecular weight, degree of methacrylation and polymer concentration, MeHA hydrogels can span a wide range of stiffness values relevant to healthy and fibrotic tissues [96].

MeHA hydrogels are particularly useful because their network properties can be tuned not only at the time of gel formation but also after cell encapsulation. Photoinitiated crosslinking strategies, including spatial patterning and sequential polymerization, enable user-directed increases in stiffness over time, thereby mimicking the evolving mechanical environment that characterizes wound repair and fibrosis. In parallel, the introduction of MMP-cleavable crosslinks allows cells to generate traction and reorganize their local microenvironment in a way that is difficult to reproduce in static non-degradable matrices [79], [96].

Collectively, these properties position HA- and MeHA-based hydrogels as powerful platforms for modelling wound healing and fibrosis *in vitro*, combining biological relevance with tunable mechanics and compatibility with spatially and temporally controlled modifications [79], [81], [96].

Despite their versatility, many MeHA-based mechanobiology platforms reported in literature have been implemented primarily in two-dimensional culture formats,

where changes in stiffness can be precisely controlled, but three-dimensional features of native tissues (*e.g.* spatial confinement, heterogeneous architecture and 3D force transmission) are not fully recapitulated. In addition, several *in vitro* wound healing and fibrosis studies rely on immortalized or murine-derived cell models, which can limit direct translation to human biology. These limitations motivate the development of three-dimensional MeHA systems that combine dynamic control of mechanics with architectures that better approximate tissue microenvironments and that can be integrated with primary human immune-stromal components. Rather than relying on MMP-cleavable linkers, where cell-driven degradation becomes a necessary prerequisite for spreading and may obscure stiffness-driven effects, MeHA can be implemented as macroporous granular hydrogels, in which cells occupy interconnected void spaces between microgels and can spread without requiring extensive network degradation. For these reasons, this thesis develops a three-dimensional, phototunable MeHA granular hydrogel platform that supports static and dynamically stiffened conditions, enabling mechanobiology studies in more physiologically relevant microenvironments.

## 2 Aim of the work

Dysregulated wound healing can progress toward fibrosis and impaired tissue function, motivating the development of advanced *in vitro* models that enable controlled investigation of how extracellular matrix (ECM) mechanics regulate cell behavior. While two-dimensional culture models have provided foundational insights into mechanotransduction, they poorly capture key aspects of tissue repair, including three-dimensional force transmission and the temporal evolution of ECM mechanics. Moreover, in many three-dimensional models, small pore size and limited remodeling can impose geometric constraints that confound stiffness-dependent responses by physically restricting cell spreading and migration. These limitations motivate three-dimensional platforms that enable dynamic control of stiffness while also helping to decouple matrix stiffness from cell confinement.

The overall aim of this thesis was to contribute to the development of a mechanically tunable three-dimensional hydrogel-based *in vitro* system to investigate cellular mechanisms relevant to wound healing and fibrosis using relevant human primary cell types. The dermis was selected as a reference tissue because skin undergoes wound healing and fibrosis, dermal mechanics are well characterized and primary dermal fibroblasts can be obtained from surgical discard tissue. This provides a practical and biologically relevant context to define physiologically meaningful stiffness targets and to study how stromal and immune cells respond to changes in matrix mechanics.

A first objective was to optimize the synthesis of methacrylated hyaluronic acid (MeHA), the backbone polymer used to fabricate the hydrogel network. Although HA functionalization strategies are widely reported, achieving reproducible degrees of methacrylation remains technically challenging and strongly influences hydrogel stiffness, degradability and crosslinking efficiency. Therefore, the synthesis protocol was optimized to improve batch-to-batch reproducibility and generate sufficient material for subsequent experiments. The synthesized MeHA was characterized to quantify the degree of methacrylation and to assess whether the target functionalization was achieved.

A second major objective was to fabricate and characterize MeHA hydrogels spanning stiffness values relevant to healthy and fibrotic dermal tissue and to establish a chemistry that enables *in situ* stiffening. Hydrogels were fabricated via Michael-type addition and subsequently stiffened through a secondary photoinduced polymerization step using a cytocompatible photoinitiator. Rheological measurements were used to quantify the mechanical properties of static “soft” and “stiff” formulations and dynamically stiffened conditions, establishing a mechanically defined framework to study how stiffness magnitude and mechanical history regulate cell behavior.

A third objective was to implement a macroporous granular architecture to better decouple matrix stiffness from geometric confinement effects that can arise in dense hydrogel networks. Bulk hydrogels were mechanically fragmented to generate microgels which were assembled into granular hydrogels with interconnected void space, providing a three-dimensional matrix that is permissive to cell organization and local remodeling. To support cell-based studies within this granular network, a microfluidic device was fabricated to retain and spatially localize the granular hydrogels. This enabled controlled cell co-loading and provided compatibility with routine culture workflows including media exchange, immunofluorescence staining and fluorescence imaging.

Finally, experimental methods for the future inclusion of human macrophages in the platform were established. Primary monocytes were isolated from whole blood and validated by flow cytometry. This provided the technical basis for macrophage differentiation and integration into the hydrogel platform to study macrophage-fibroblast crosstalk under defined and evolving mechanical conditions.

Although dermal wound healing was used as the reference context, the platform design is intended to be adaptable to other fibrotic tissue models by tuning matrix mechanics and incorporating tissue-relevant stromal and immune cell populations.

## 3 Materials and methods

### 3.1. Material synthesis

Methacrylated hyaluronic acid (MeHA) synthesis was performed following standard laboratory safety procedures and under a chemical fume hood to ensure safe and controlled conditions.

#### 3.1.1. MeHA synthesis

The synthesis of MeHA was adapted from Caliri *et al.* [89], [97]. Sodium Hyaluronate (60 kDa HA, Lifecore BIOMEDICAL, 030433) was dissolved in Milli-Q water (Milli-Q, Merck, PM3AB08017) at a concentration of 2g of HA per 100 mL of H<sub>2</sub>O. In particular, half of the total water volume was first added to a round bottom flask, HA was then slowly added using a plastic spatula and the remaining water was added to reach the final volume. The solution was stirred overnight at 700 rpm using a magnetic stirrer (Heidolph Instruments).

The following day, the pH of the HA solution was measured using pH paper or pH meter (pH 1100L, VWR) depending on the volume. After dissolution, the pH was typically around 5 and it was increased to pH 8 by adding 1M NaOH dropwise using a glass Pasteur pipette. Methacrylic anhydride (Sigma-Aldrich, 276685-100ML) was then added to the reaction mixture at 5.6 mL/1 g of HA with a syringe to the reaction solution. In particular 1 ml of methacrylic acid was added every 20-30 minutes until the final required volume was added.

Since the reaction is exothermic, the flask was placed in an ice bath (wide beaker filled with ice) on the stirring plate to maintain a low temperature during the reaction.

To promote efficient functionalization, the pH was maintained between 8 and 9 through the reaction to favour partial deprotonation of HA hydroxyl groups and thereby support reaction with methacrylic anhydride (Figure 3.1). The pH was measured every 10-20 minutes and adjusted as need by adding 1M or 0.1M NaOH dropwise. The reaction proceeded for 8-10 hours, with ice replaced every 2 hours and pH adjusted every 10-20 minutes. Then the mixture was left stirring overnight on ice.

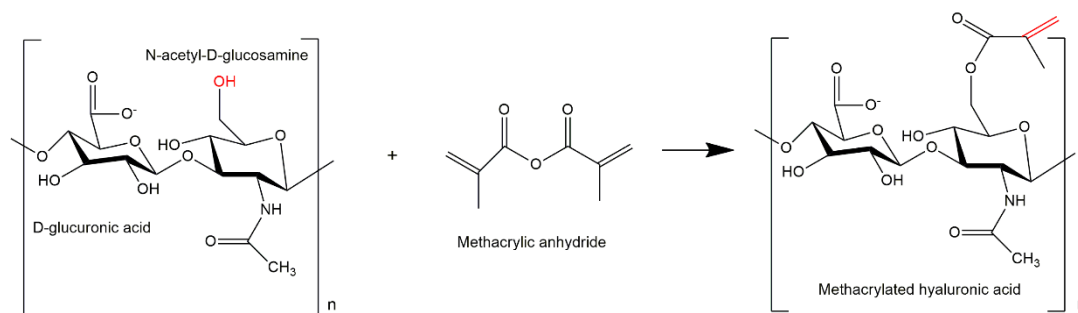


Figure 3.1: Synthesis of methacrylated hyaluronic acid (MeHA). Methacrylic anhydride reacts with primary hydroxyl groups on the hyaluronic acid backbone to introduce pendant methacrylate groups (highlighted in red). These methacrylate functionalities provide polymerizable sites used for subsequent hydrogel fabrication [97].

### 3.1.2. Dialysis

The morning after the reaction, the product was purified to remove residual reagents and reaction by-products. The reaction mixture was transferred to 50 mL conical tubes and centrifuged (Eppendorf Centrifuge 5430) for 5 minutes at 3000 rpm. The supernatant containing functionalized HA was collected and transferred into dialysis tubing (Spectra/Por, 132665T; MWCO 6-8 kDa), leaving sufficient headspace to accommodate swelling of the polymer. Dialysis was performed at room temperature for 7 days in a 5 L beaker containing DI water under stirring. The DI water was replaced twice on the first day and subsequently every 24 h to maintain the concentration gradient between the dialysis buffer and the sample.

### 3.1.3. Lyophilization

After dialysis, the product was collected in 50 mL conical tubes and frozen overnight in an ultra-low temperature freezer (BINDER). Samples were then lyophilized (Lyovapor L-300, BUCHI) for 3 days. During lyophilization, tube lids were removed and a Kimwipe was placed over each tube to prevent loss of material. Lyophilization was considered complete when no residual ice was visible in the samples.

## 3.2. Chemical characterization

$^1\text{H}$ -NMR spectroscopy was performed to quantify the degree of methacrylation of the HA backbone. Measurements were acquired on a high-resolution NMR spectrometer (Bruker Ascend Aeon 400) at the Department of Environmental Systems Science, ETH Zürich.

### 3.2.1. Sample preparation for NMR characterization

Lyophilized MeHA (5 mg) was weighed (Ohaus Pioneer) and dissolved in 500  $\mu\text{L}$  deuterium oxide ( $\text{D}_2\text{O}$ ; Sigma-Aldrich, 151882) in a glass vial. The solution was briefly vortexed to support dissolution and then transferred into an NMR tube (DWK Life Sciences, 00141126).

### 3.2.2. NMR characterization and spectral processing

Spectra were acquired with a total acquisition time of 1 hour and 30 minutes and 1024 scans. Data were processed using MestReNova (Mestrelab Research).

An automatic phase correction was applied, followed by baseline correction using a Bernstein polynomial fit (polynomial order 3). The  $\text{D}_2\text{O}$  solvent peak at 4.79 ppm was used as a reference to support consistent comparison between spectra.

The degree of methacrylation was quantified as described by Smeds *et al.* [98] by integrating the vinyl proton peaks associated with methacrylate groups (5.6-5.9 ppm and 6.1-6.4 ppm) and normalizing these to signals from the HA backbone (3.0-4.6 ppm).

## 3.3. Pooling

To reduce batch-to-batch variability, multiple MeHA batches (~10 g of lyophilized product in total) were pooled. Lyophilized MeHA was dissolved overnight in Milli-Q water at 2 g per 100 mL under vigorous stirring. The solution was dialyzed (Spectra/Por, 132665T; MWCO 6–8 kDa) for 2 days.

The dialyzed product was then sterilized by filtration using a 0.45  $\mu\text{m}$  filter (Fisherbrand, FB12566501) under a biological safety cabinet (Telstar, EN-12469-2000). The filtered solution was frozen at  $-80\text{ }^{\circ}\text{C}$ , lyophilized, and the resulting lyophilized MeHA was stored at  $-80\text{ }^{\circ}\text{C}$  until further use.

## 3.4. Hydrogel preparation

MeHA hydrogels were prepared using the pooled MeHA batches synthesized as described in Section 3.1.1. Bulk MeHA hydrogels were formed via Michael-type addition and subsequently subjected to a secondary photoinitiated crosslinking step as described by Caliarì *et al.* [86]. Unless otherwise stated, all hydrogel preparation

steps intended for cell culture were performed under sterile conditions in a biological safety cabinet using sterilized tools (*e.g.* autoclaved tweezers and spatulas).

### 3.4.1. Bulk hydrogel fabrication by Michael-type addition

Sterile MeHA was dissolved in sterile phosphate buffered saline solution (PBS, Gibco) to obtain a stock solution of 60 mg/mL. The required mass of MeHA was weighed, PBS was added and the tube was vortexed (Scientific Industries, G560E) for 2 hours to promote dissolution and mixing. The solution was then sonicated (Branson, CPX2800H-E) for 1-2 hours to reduce trapped air bubbles and stored at 4 °C overnight.

Bulk hydrogels were fabricated the following day. Sigma-coated glass coverslips were sprayed with ethanol and 500  $\mu\text{m}$  rubber spacers were sterilized with 70% ethanol. Two spacers were placed on each coverslip to define the hydrogel thickness; after preparation, the hydrogel precursor solution was pipetted onto the bottom coverslip and covered with a second coverslip.

Hydrogel precursor solutions were prepared in 1.5 mL Eppendorf tubes by mixing the components in a defined order. The formulations (total volume 700  $\mu\text{L}$  per condition) are reported in Table 1 and were designed to yield four replicates for bulk rheological characterization (40  $\mu\text{L}$  each) and two larger bulk hydrogels (250  $\mu\text{L}$  each) for subsequent preparation of granular microgels (Section 3.4.3).

Table 1: Formulation used to prepare 3% (w/v) MeHA bulk hydrogels for each thiol:methacrylate ratio. Volumes are reported in  $\mu\text{L}$  (total volume 700  $\mu\text{L}$  per formulation). The final RGD concentration (1.17 mM) was kept constant across formulations; for ratios 1:3 and 1:7, a different RGD stock concentration was used, so the added RGD volume was adjusted accordingly.

|             | 3%<br>MeHA | DI water | 1.17 mM<br>RGD | 0.05 M<br>TEOA | PEG(SH) <sub>2</sub> |
|-------------|------------|----------|----------------|----------------|----------------------|
| <b>1:1</b>  | 350        | 82.76    | 4.43           | 35             | 227.81               |
| <b>1:2</b>  | 350        | 196.67   | 4.43           | 35             | 113.90               |
| <b>1:3</b>  | 350        | 234.12   | 4.94           | 35             | 75.94                |
| <b>1:4</b>  | 350        | 253.62   | 4.43           | 35             | 56.95                |
| <b>1:6</b>  | 350        | 272.6    | 4.43           | 35             | 37.97                |
| <b>1:7</b>  | 350        | 277.52   | 4.94           | 35             | 32.54                |
| <b>1:8</b>  | 350        | 282.1    | 4.43           | 35             | 28.47                |
| <b>1:9</b>  | 350        | 285.27   | 4.43           | 35             | 25.3                 |
| <b>1:10</b> | 350        | 287.79   | 4.43           | 35             | 22.78                |

For each formulation, 350  $\mu\text{L}$  of MeHA solution was first combined with the required volume of DI water and vortexed for  $\sim 30$  s. RGD peptide was then added to enable cell adhesion, followed by vortexing for  $\sim 30$  s. Triethanolamine (TEOA, Thermo Scientific) was added using a viscous pipette (Microman E, Gilson) to buffer the precursor solution, followed by vortexing for  $\sim 30$  s. Finally, PEG(SH)<sub>2</sub> was added to initiate gelation and the mixture was vortexed a final time for  $\sim 30$  s.

Hydrogel precursor solutions were cast by pipetting four 40  $\mu\text{L}$  samples (for rheology) and two 250  $\mu\text{L}$  samples (for fragmentation) between spacer-defined coverslip structures. Samples were allowed to crosslink at room temperature for 2 h. After crosslinking, the top coverslip was removed and the hydrogels were transferred to tissue culture dishes (TPP, 22.1  $\text{cm}^2$  growth area) containing 7 mL PBS to allow swelling. Dishes were sealed with Parafilm (Sigma-Aldrich) and stored at 4  $^{\circ}\text{C}$  overnight.

### 3.4.2. Secondary crosslinking by photoinitiated free-radical polymerization

To introduce additional covalent crosslinks after the initial network formation, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; synthesized previously in the laboratory) was used as a photoinitiator to enable photoinitiated free-radical polymerization of remaining methacrylate groups.

LAP solutions (0.01% and 0.1% w/v) were prepared in PBS. Bulk hydrogels were incubated in the LAP solution for 30 min in a 6-well plate protected from ambient light (covered with aluminum foil). Hydrogels were then placed on the rheometer stage and exposed to 405 nm light at 10  $\text{mW}/\text{cm}^2$  for 10 min to activate LAP and generate radicals.

### 3.4.3. Granular hydrogel fabrication by mechanical sieving

Following overnight swelling, bulk hydrogels were mechanically fragmented to generate granular microgels using a syringe-based sieving approach adapted from Kessel *et al.* [99].

For each condition, 500  $\mu\text{L}$  of swollen bulk hydrogel was pushed through a 2 mL syringe (B. Braun, 4606701V) fitted with a 90  $\mu\text{m}$  mesh into a 2 mL Eppendorf tube. Growth medium (DMEM, Gibco, supplemented with 10% FBS (Gibco, A3160801) and 1% penicillin/streptomycin (Thermo Fisher Scientific, 10378016)) was used to rinse the syringe walls. The sieving step was repeated a total of three times. Fragmented hydrogels were then distributed into two 2 mL tubes, topped up with growth medium and stored at 4  $^{\circ}\text{C}$  until use.

### 3.5. Hydrogel rheological characterization

Hydrogels were characterized using a rheometer (Anton Paar MCR 502) equipped with an 8 mm sandblasted parallel-plate geometry. Motor adjustment and torque calibration were performed prior to measurements. Oscillatory measurements were used to determine the storage modulus ( $G'$ ), loss modulus ( $G''$ ) and normal force under defined testing conditions.

To minimize dehydration during rheological testing, water soaked Kimwipes were placed around the measurement stage to maintain a humid local environment during acquisition.

#### 3.5.1. Bulk hydrogel characterization

After overnight swelling in PBS, hydrogels were removed from the dish and punched using an 8 mm biopsy punch (Mayhew Pro 66010) to match the geometry diameter. Measurements were performed at 37 °C using the Peltier-controlled stage. The storage shear modulus ( $G'$ ) and loss shear modulus ( $G''$ ) were measured using an oscillatory time sweep at constant strain amplitude  $\gamma = 0.5\%$  and angular frequency  $\omega = 1$  rad/s for 180 s.

#### 3.5.2. *In situ* stiffening characterization of bulk hydrogels

For *in situ* stiffening measurements, the rheometer was equipped with a glass stage enabling illumination from below. A 405 nm LED light source (Thorlabs, M405L4-C1) was coupled through an adjustable collimator adapter (Thorlabs, SM2F32-A) and a light guide fixed to the underside of the glass stage. Light intensity was controlled with an LED driver (Thorlabs, LEDD1B) and calibrated using a photodiode power sensor (Thorlabs, S120VC) to achieve 10 mW/cm<sup>2</sup> at the sample plane.

Because the glass stage does not include Peltier temperature control, temperature was maintained at 37 °C using a circulating water bath system (Anton Paar, Viscotherm VT 2).

Hydrogels (8 mm diameter) were incubated for 30 minutes in LAP solution (PBS; concentrations as specified in Section 3.4.2) and then placed on the rheometer stage. Oscillatory time sweeps were performed at  $\gamma = 0.5\%$  and  $\omega = 1$  rad/s, consisting of 180 s baseline measurement (pre-stiffening), 600 s illumination at 10 mW/cm<sup>2</sup> and a further 180 s post-illumination measurement.

After the *in situ* test, samples were swollen in PBS for 2 hours and subsequently re-measured (Section 3.5.1) to assess storage shear modulus ( $G'$ ) and loss shear modulus ( $G''$ ) after swelling following secondary crosslinking.

### 3.5.3. Granular hydrogel characterization

Granular hydrogels prepared as described in Section 3.4.3 were stored at 4 °C until testing. Measurements were performed at 37 °C using the Peltier-controlled stage. The oscillatory measurement conditions matched those used for bulk hydrogels ( $\gamma = 0.5\%$ ,  $\omega = 1$  rad/s), unless otherwise specified.

#### 3.5.3.1. Sample preparation: first method

Granular hydrogel suspensions in 2 mL tubes were centrifuged at 18000 rcf for 20 minutes to separate the granular phase from the supernatant. The supernatant was removed and 200  $\mu$ L of granular phase was transferred into a 1.5 mL tube and diluted with 200  $\mu$ L growth medium. The tube was centrifuged again at 18000 rcf for 20 minutes to promote jamming. Before loading onto the rheometer, the supernatant was removed and 50  $\mu$ L of jammed granular material was collected using a viscous pipette. The plate gap was set to 1.0 mm and the geometry was unlocked during loading to help covering all the geometry area.

#### 3.5.3.2. Sample preparation: second method

Granular hydrogel suspensions in 2 mL tubes were centrifuged at 18000 rcf for 20 minutes. After removal of the supernatant, 200  $\mu$ L of granular phase was transferred to a 1.5 mL tube and centrifuged at 18000 rcf for 10 minutes to promote jamming and remove residual medium. Supernatant was removed using a P10 pipette and remaining liquid was absorbed using a Kimwipe. With the geometry unlocked and the gap set to 1.0 mm, the sample was loaded onto the stage using a viscous pipette. The gap was then reduced to 0.9 mm and residual liquid at the edge was removed with a Kimwipe prior to starting the measurement.

### 3.5.4. Data analysis

Raw rheology data were exported and processed in Excel (Microsoft 365). For each sample,  $G'$  values were averaged after reaching a stable plateau to obtain a representative stiffness value. Processed data were plotted in GraphPad Prism 10.6.1 (GraphPad Software, San Diego, CA).

## 3.6. Microfluidic device fabrication

Polydimethylsiloxane (PDMS) microfluidic devices (Figure 3.2) were fabricated in the Ehrbar Laboratory at Universitätsspital Zürich using standard soft-lithographic techniques. In particular, master molds previously developed by Krattiger *et al.* were used to replicate the device geometry [100].

### 3.6.1. Soft lithography

PDMS base and curing agent (Elastosil RT 601 A/B, Ameba AG) were mixed at a 9:1 ratio (w/w) using a mechanical mixer (Thinky ARM-310, Intertronics) for 3 minutes at 2000 rpm. The mixture was then poured onto the master molds, ensuring complete coverage of the microstructured features and sufficient thickness for mechanical stability. Air bubbles were removed by degassing the molds in a desiccator. PDMS was cured in an oven at 70 °C for 1 hour.

After curing, the PDMS layer was carefully peeled off the mold and individual devices were cut using a scalpel. Inlet and outlet holes (two per channel) were created using a biopsy punch (20G Catheter Punch; Schmidt Press).

### 3.6.2. Plasma treatment and glass bonding

PDMS devices and round glass coverslips (25 mm diameter, #1.5 thickness; Marienfeld) were cleaned to remove dust and treated with oxygen plasma for 60 seconds (Diener, Plasma Cleaner Zepto One). Plasma treatment replaces surface methyl ( $-CH_3$ ) groups on PDMS with reactive silanol ( $-OH$ ) groups and increases hydroxylation of the glass surface, enabling irreversible bonding. Immediately after plasma exposure, PDMS and glass were aligned and brought into contact, applying gentle pressure with tweezers while avoiding trapped air bubbles.

Bonded devices were then placed on a hot plate at 80 °C for 1 hour to strengthen the bond. Prior to use in cell experiments, devices were sterilized and stored to maintain sterility.



Figure 3.2: Representative picture of the fabricated PDMS microfluidic device bonded to a glass coverslip, shown during loading via pipette [100].

### 3.7. Co-loading of granular hydrogels and cells in a microfluidic device

Granular hydrogels were co-loaded with neonatal human dermal fibroblasts into microfluidic devices fabricated as described in Section 3.6 to enable a three-dimensional culture configuration within the confinement chamber.

#### 3.7.1. Fibroblast cell culture

Neonatal human dermal fibroblasts (NHDF) were cultured in Growth Medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich) and 1% Penicillin-Streptomycin (P/S, Gibco), cultured in incubator (BINDER, 9040-0112) at 37 °C and 5% CO<sub>2</sub>. All manipulations and operations were performed in sterile conditions under biological safety hood.

Cell passaging was performed when cells were at around 60-70% confluency to maintain healthy fibroblast cultures and ensure optimal growth conditions. Trypsin-EDTA (Thermo Fisher, 25200056) and growth medium were pre-warmed to 37 °C in a bead bath (Grant Instruments, U42351001) before use. At first the old medium was aspirated from the flask (T150), followed by a rinse with 12 mL of PBS-/- to remove any residual serum proteins that could inhibit trypsin activity. The PBS-/- was then aspirated before adding 5 mL of trypsin to detach the cells by cleaving adhesion proteins. The flask was incubated at 37 °C for approximately 3 minutes and then the flask was gently tapped to help cell detachment. To neutralize the trypsin, 5 mL of pre-warmed growth medium was added to the flask. Then the cell suspension was transferred to a conical tube and the appropriate volume of cells was taken depending on the desired split ratio after counting them. Cells were centrifuged at 300 rcf for 5 minutes. Following centrifugation, the supernatant was carefully aspirated, leaving the cell pellet intact at the bottom of the tube. Then the pellet was resuspended in 12 mL of fresh growth medium by gentle pipetting to ensure an uniform suspension without clumps. Finally, cells were transferred into a new flask and an additional 13 mL of growth was added to bring the total volume to 25 mL.

#### 3.7.2. Device loading

Granular hydrogels were prepared as described in chapter 3.4.3 and then centrifuged with the same method used for the rheological characterization (chapter 3.5.3.2).

A 6-well plate was used and each well was pre-treated with 3 µL of PBS-/- to improve adhesion to the bottom of the plate. One microfluidic device was placed in each well, with the inlets oriented toward the right side to facilitate handling.

For each experimental condition, a fresh Eppendorf tube was used to prepare the gel-cell mixture. First, 5% (w/v) methylcellulose was added, followed by the jammed

granular hydrogel. The mixture was briefly vortexed to ensure homogeneous mixing and in parallel cells were counted and resuspended to obtain a final concentration of approximately  $24.6\text{--}30 \times 10^6$  cells/mL. As a standard formulation for a 100  $\mu\text{L}$  gel-cell mixture, 75  $\mu\text{L}$  of 5% methylcellulose was combined with 8.3  $\mu\text{L}$  of granular gel and 17  $\mu\text{L}$  of cell suspension at the desired seeding density.

The appropriate volume of concentrated cell suspension was added to each gel-methylcellulose mixture and vortexed to achieve uniform cell distribution. Subsequently, 40  $\mu\text{L}$  of the resulting suspension was carefully loaded into the confinement chamber of each microfluidic device (Figure 3.3).

Loading was visually monitored under a microscope (EVOS M5000) to confirm sufficient filling and jamming of the channel. If the confinement chamber was not fully occupied, additional gel-cell suspension was added until adequately filled. The inlets and outlets were then sealed using sterile tungsten wires previously prepared and sterilized.

After 10 minutes, 100  $\mu\text{L}$  of growth medium was gently flushed through both media channels of each device using a pipette. The 6-well plate containing the devices was then transferred to an incubator at 37 °C. After 2 hours, the medium was refreshed by flushing an additional 100  $\mu\text{L}$  of fresh medium through both channels. To maintain humidity and prevent evaporation, 1 mL of sterile PBS $^-$  was added to the inter-well spaces of the plate. The plate was then returned to the incubator overnight.

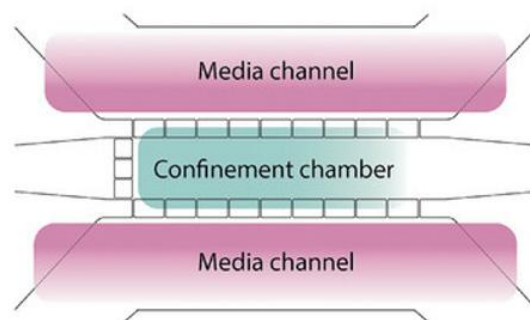


Figure 3.3: Schematic of the microfluidic device layout, consisting of a central confinement chamber and two adjacent media channels for medium perfusion and exchange. The confinement chamber is 1 mm wide and uses 490  $\mu\text{m}$ -long pillars spaced 30  $\mu\text{m}$  apart [100].

### 3.7.3. Immunofluorescence staining

The day after, fixation of the devices was performed. 100  $\mu\text{L}$  of sterile PBS $^{+}$  (Sigma-Aldrich) was gently flushed through both media channels of each device to remove residual growth medium. Subsequently, 100  $\mu\text{L}$  of freshly prepared 4% paraformaldehyde (PFA, Thermo Fisher) was introduced into each media channel and

incubated at room temperature for 30 minutes to ensure effective fixation. Then the channels were rinsed by flushing 100  $\mu$ L of PBS+/+ through each channel. To keep the devices hydrated, 1 mL of PBS-/- was added to the inter-well spaces of the 6-well plate containing the devices and the plate was sealed with Parafilm and stored at 4 °C until further analysis.

To permeabilize cell membranes and reduce nonspecific binding, 100  $\mu$ L of permeabilization buffer composed of 0.25% Triton X-100 (Sigma-Aldrich) and 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS+/+ was gently introduced into each media channel. Samples were incubated for 45 minutes at room temperature.

In parallel, a phalloidin staining solution was prepared by diluting fluorescently labelled phalloidin (Phalloidin 555, Abcam) 1:400 in PBS+/+ containing 1% BSA. After permeabilization, the buffer was removed and 100  $\mu$ L of the phalloidin solution was added per each channel. The plates were wrapped with Parafilm to prevent evaporation and incubated overnight at 4 °C.

The following day, the phalloidin solution was removed and samples were washed three times with PBS+/+, with 30 minutes of waiting time in between them at room temperature to ensure removal of unbound dye.

For nuclear staining, a DAPI solution (4',6-diamidino-2-phenylindole, Thermo Fisher) was prepared at a final dilution of 1:1000 in PBS+/+. Then, 100  $\mu$ L of the solution was added to each media channel of the microfluidic devices. Samples were incubated for 30 minutes at room temperature.

Following incubation, samples were washed three additional times with PBS+/+, allowing 15 minutes between each wash to remove excess DAPI. After the final wash, samples were left covered with a small volume of PBS+/+ to maintain hydration.

### 3.7.4. Fluorescence imaging

Brightfield imaging to verify the confinement chamber filling was performed using an inverted fluorescence microscope (EVOS™ M5000 Imaging System, Thermo Fisher Scientific) controlled with EVOS2 software using 4 $\times$ , 10 $\times$  and 20 $\times$  objectives.

Fluorescent imaging of fixed and stained samples was performed on a THUNDER Imager Cell microscope (Leica Microsystems) using 5 $\times$ , 10 $\times$  and 20 $\times$  objectives. For 20 $\times$  acquisitions, epifluorescence z-stacks were collected across the thickness of the confinement chamber. Nuclei (DAPI) were imaged using 390 nm excitation (30% illumination intensity) and F-actin (phalloidin 555) was imaged using 555 nm excitation (30% illumination intensity) with a 565-615 nm emission bandpass filter. Images were acquired as multichannel stacks (typically 1024  $\times$  1024 pixels per frame). The lateral pixel size was taken from the image metadata (e.g. 0.646  $\mu$ m/pixel) and the

z-stack depth and number of optical sections were adapted per field of view to capture the full thickness of the cell-populated region.

### 3.7.5. Image processing and visualization

Image processing was performed in Fiji (ImageJ). Images were imported together with their acquisition metadata, including pixel size ( $\mu\text{m}/\text{pixel}$ ) and z-stack information. For qualitative visualization, brightness/contrast were adjusted uniformly within the same experimental condition. Multichannel stacks were split/merged using the Channels tool. Maximum intensity projections (MIPs) were generated from z-stacks to visualize cell distribution and cytoskeletal organization across the full z-range. Scale bars were added based on the pixel size reported in the image metadata and identical scale settings were used within each figure panel to enable comparison between fields of view.

## 3.8. Human monocytes isolation from whole blood

After approval by the Kantonale Ethikkommission Zürich, coded human whole blood samples were obtained from Blutspende SRK Schweiz as residual material from routine blood donations.

All the work was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP).

### 3.8.1. PBMC isolation

EDTA-treated whole blood was ordered one week in advance and delivered at 20 °C. Ficoll-Paque (Sigma-Aldrich) and PBS–/– were equilibrated to 20 °C prior to use to ensure reproducible density separation.

Upon arrival, 15 mL of whole blood were transferred into 50 mL conical tubes and diluted 1:1 (v/v) with PBS–/–. The diluted blood was mixed by gentle inversion. 15 mL of Ficoll-Paque were added to new 50 mL conical tubes and 30 mL of diluted blood were carefully layered on top of the Ficoll-Paque by pipetting against the tube wall to avoid mixing. Samples were centrifuged at 800 rcf for 30 minutes at 20 °C with the brakes off. After centrifugation, the PBMC layer at the plasma-Ficoll interface was collected using a serological pipette and transferred to a new tube (Figure 3.4)

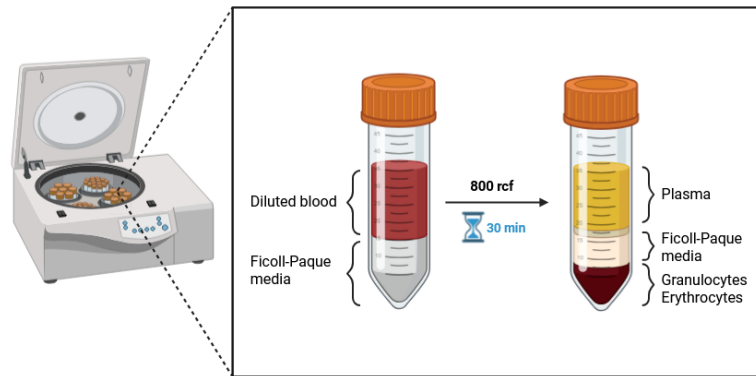


Figure 3.4: Schematic of the PBMCs isolation by Ficoll-Paque density gradient centrifugation. Diluted whole blood is layered onto Ficoll-Paque and centrifuged to separate plasma, PBMCs (grey interphase) and granulocytes/erythrocytes. Created with Biorender.com.

Collected PBMCs were washed by filling the tube to 50 mL with PBS<sup>-/-</sup> and centrifuging at 300 rcf for 8 minutes. The supernatant was aspirated and washing was repeated until the supernatant appeared clear. PBMCs were pooled in PBS<sup>-/-</sup> and counted using trypan blue with an automated cell counter (NanoEntek EVE) and counting slides (NanoEntek, EVS-050).

### 3.8.2. Monocyte negative immunomagnetic isolation

One day prior to isolation, solution medium was prepared (PBS<sup>-/-</sup> supplemented with 2% FBS and 1 mM EDTA) under sterile conditions. The negative immunomagnetic selection of the monocyte population was performed consequently after the PBMCs isolation from whole blood as recommended in the datasheet from StemCell Technologies.

PBMCs were resuspended at  $5 \times 10^7$  cells/mL of solution medium in 5 mL round-bottom polystyrene tubes. The Isolation Cocktail and Platelet Removal Cocktail were added at 50  $\mu$ L/mL, mixed and incubated for 5 minutes at room temperature. Magnetic nanoparticles were vortexed for 30 s to ensure dispersion and added at 50  $\mu$ L/mL, followed by 5 minutes of incubation at room temperature. Samples were brought to a final volume of 2.5 mL with PBS<sup>-/-</sup> and mixed by gentle pipetting.

Tubes (without caps) were placed in an EasyEights™ EasySep™ magnet for 2.5 minutes at room temperature. The enriched cell suspension was carefully transferred to a new tube and subjected to a second magnetic separation step. Enriched cells were collected, counted using trypan blue and processed for characterization.

### 3.8.3. Flow cytometry staining

Flow cytometry staining was performed to assess monocyte viability and to quantify CD14/CD16 expression after negative immunomagnetic enrichment.

After isolation, cells were centrifuged at 400 rcf for 5 minutes at 4 °C and resuspended in PBS<sup>-/-</sup>. Cells were transferred to round-bottom polystyrene tubes at a concentration of  $0.5 \times 10^6$  cells/100  $\mu$ L and kept on ice.

For viability assessment, a live/dead stain (LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit, for 405 nm excitation, Thermo Fischer) was included. To generate a positive control, half of the cells in the live/dead-only control tube were heat-killed by incubation at 65 °C for 1 minute, followed by immediate cooling on ice for 1 minute before recombination with the live cell fraction. Live/dead staining was performed by incubating cells with the viability dye diluted in PBS<sup>-/-</sup> (1  $\mu$ L dye per  $1 \times 10^6$  cells) for 20 minutes on ice in the dark. Serum-containing buffers were avoided during this step to prevent interference with the viability dye. After staining, samples were topped up to 1 mL with PBS<sup>-/-</sup> and centrifuged at 400 rcf for 5 minutes at 4 °C.

To reduce nonspecific binding, cells were incubated with Fc receptor blocking solution (BioLegend) diluted in FACS buffer (eBioscience™ Flow Cytometry staining buffer, Invitrogen) (5  $\mu$ L per  $1 \times 10^6$  cells) for 10 minutes at room temperature.

Surface antibody staining was performed by incubating cells in 100  $\mu$ L of antibody solution for 30 minutes at room temperature in the dark. Antibodies against CD14 (10  $\mu$ L/100  $\mu$ L, CD14 Monoclonal Antibody FITC, Invitrogen) and CD16 (2  $\mu$ L/100  $\mu$ L, CD16 Monoclonal Antibody Alexa Fluor™ 647, Invitrogen) were used. After incubation, cells were washed twice with FACS buffer by centrifugation at 400 rcf for 5 minutes. Stained cells were resuspended in 300  $\mu$ L of FACS buffer and stored at 4 °C in the dark until acquisition.

Single-stained reference controls for spectral unmixing were prepared using compensation beads (UltraComp eBeads™ Compensation Beads, Invitrogen). Beads were vortexed thoroughly and one drop was added per tube. Separate bead tubes were stained with each fluorophore-conjugated reagent used in the experiment (CD14-FITC and CD16-Alexa Fluor 647) and incubated on ice for 20 minutes in the dark. Beads were washed with flow cytometry staining buffer, centrifuged at 600 rcf for 5 minutes and resuspended in 300  $\mu$ L staining buffer prior to acquisition. Two unstained controls were prepared in staining buffer: one containing cells only (unstained cell control) and one containing compensation beads only (unstained bead control).

### 3.8.4. Flow cytometry acquisition

Flow cytometry measurements were performed at the Flow Cytometry Core Facility (FCCF) at ETH Zürich using a Cytex Aurora spectral flow cytometer (Cytex

Biosciences). The acquisition was designed to quantify cell viability (LIVE/DEAD Violet) and monocyte subset distribution based on CD14 and CD16 surface expression.

Spectral unmixing was performed using single-stained reference controls acquired during the same experimental session and applied automatically by the acquisition software prior to downstream data analysis. All samples were acquired under comparable instrument settings within and across different experiments to enable consistent comparison of CD14/CD16 quadrant distributions.

### 3.8.5. Flow cytometry data analysis

Flow cytometry data were analyzed using FlowJo software (version 10, BD Biosciences). Data were gated to quantify the distribution of CD14/CD16 subsets within viable single cells.

The gating strategy was applied in the following order: selection of the main cell population based on forward and side scatter (FSC-H vs SSC-H) to exclude debris, singlet selection (FSC-A vs FSC-H) and live cell selection by exclusion of LIVE/DEAD Violet-positive events. CD14 and CD16 positivity thresholds were defined using single-stained reference controls and then kept constant across samples to ensure consistent quadrant placement. The final output reported for each sample was the percentage of events in each CD14/CD16 quadrant (CD14<sup>+</sup>/CD16<sup>-</sup>, CD14<sup>+</sup>/CD16<sup>+</sup>, CD14<sup>-</sup>/CD16<sup>+</sup>, CD14<sup>-</sup>/CD16<sup>-</sup>) within the live singlet gate.



## 4 Results and discussion

### 4.1. MeHA synthesis

$^1\text{H}$  NMR analysis was first performed on the starting material, 60 kDa hyaluronic acid, to establish a reference spectrum prior to chemical modification (Figure 4.1). The spectrum presents characteristic peaks in the 3.0-4.6 ppm region, corresponding to the sugar ring protons of the glucuronic acid and N-acetylglucosamine units forming the HA backbone. A distinct singlet at approximately 1.9 ppm is attributed to the methyl protons of the N-acetyl group. No peaks are observed in the vinyl region (5.6-6.1 ppm) confirming the absence of methacrylate groups in the starting polymer.

All the NMR spectra presented in this section will be focused between 0-10 ppm on the x-axis to allow the reader to better capture the peak positions.

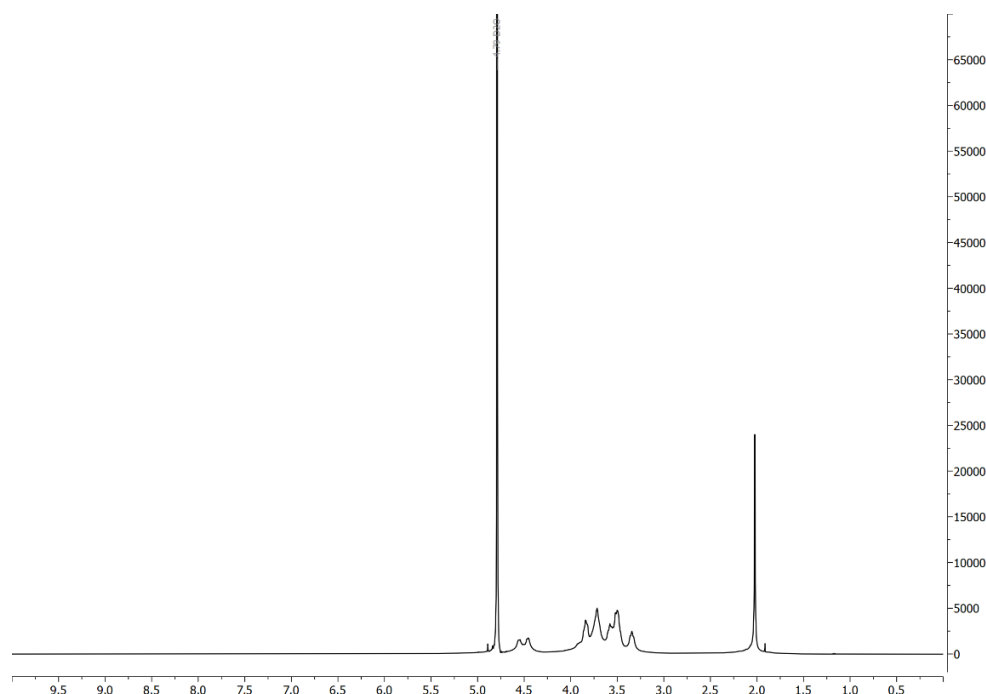


Figure 4.1:  $^1\text{H}$  NMR spectrum of 60 kDa hyaluronic acid (HA) in  $\text{D}_2\text{O}$ . Signals between 3.0-4.6 ppm correspond to HA ring protons and the singlet at  $\sim 1.9$  ppm corresponds to the N-acetyl methyl group. No resonances are present in the vinyl region (5.6-6.1 ppm), indicating absence of methacrylate groups. The residual  $\text{D}_2\text{O}$  peak (4.7 ppm) was used as a chemical shift reference.

The objective of the synthesis was to achieve a high methacrylation degree of HA (~100%, near complete substitution of the primary hydroxyl groups) to enable efficient crosslinking in subsequent hydrogel fabrication.

The degree of functionalization is a key parameter because it directly determines the number of polymerizable groups available along the HA backbone. Therefore, it affects both network formation during the Michael-type addition step and the extent of secondary photoinduced free radical crosslinking. Variations in functionalization affect hydrogel architecture and final properties including stiffness, swelling behavior and degradability [101].

As a reference for a highly functionalized MeHA (~90%), the  $^1\text{H}$  NMR spectrum reported by Caliri *et al.* is reported in Figure 4.2 [89]. In contrast to HA, the spectrum displays characteristic vinyl proton peaks at approximately 5.6 and 6.1 ppm indicating a successful methacrylation of the polymer backbone.

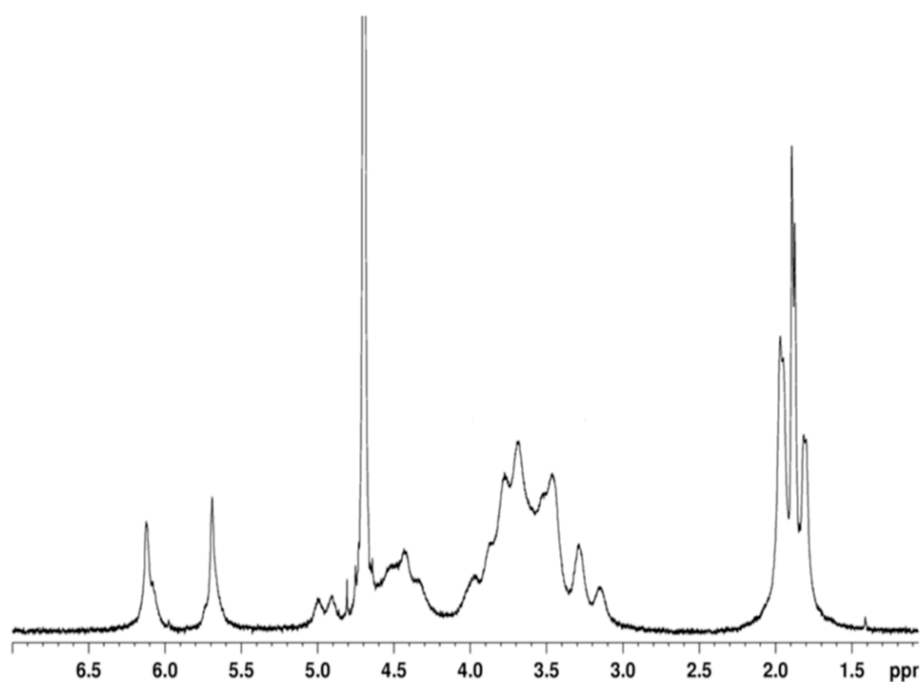


Figure 4.2: Reference  $^1\text{H}$  NMR spectrum of methacrylated hyaluronic acid (MeHA) reported by Caliri *et al.* Characteristic methacrylate vinyl proton peaks are visible at 5.6 and 6.1 ppm, consistent with successful HA methacrylation (reported functionalization ~90%) [86].

#### 4.1.1. First synthesis protocol

Figure 4.3 reports the  $^1\text{H}$  NMR spectrum obtained with the first synthesis that was performed. The presence of peaks in the vinyl region confirms the successful introduction of methacrylate groups on the HA backbone. However, peak integration

performed by normalizing the vinyl proton signal to the backbone peaks (3.0-4.6 ppm) revealed an estimated degree of functionalization of ~30%.

The synthesis was repeated under identical conditions to assess reproducibility obtaining comparable low functionalization degree. Additional  $^1\text{H}$  NMR spectra are reported in Appendix A.

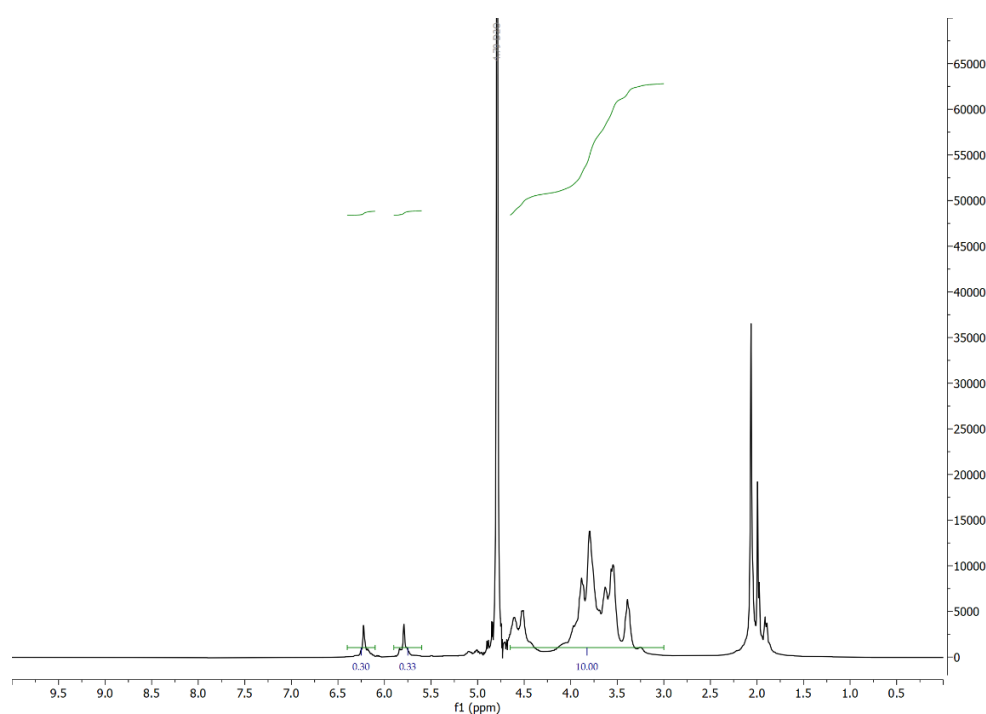


Figure 4.3:  $^1\text{H}$  NMR spectrum of MeHA from Batch I (first synthesis protocol). Vinyl proton resonances at 5.6-6.1 ppm confirm methacrylate incorporation on the HA backbone. Integration of the vinyl peaks relative to the HA backbone region (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~30% (MestReNova).

The low functionalization degree may be attributed to a combination of process-related factors. In fact, the first reactions were performed using dry ice in water instead of regular ice due to temporarily unavailability of regular ice in the laboratory. This might have caused an excessive decrease in the reaction temperature below the activation energy required for the methacrylation of the HA backbone, thereby limiting the substitution. In addition, pH adjustment was initially performed with 5 M NaOH during the early stage of the reaction (to rapidly restore pH after methacrylic anhydride addition) and then maintained with 0.1 M NaOH for the remaining reaction time. This may have generated local alkaline microenvironments which can favour methacrylic anhydride hydrolysis and reduce the fraction of reagent available to react with HA primary hydroxyl groups, ultimately lowering the final degree of methacrylation.

### 4.1.2. Second synthesis protocol

Moving forward, regular ice was used to cool the reaction mixture. pH was adjusted with 1 M NaOH in the early phase of the reaction and with 0.1 M NaOH during the remaining reaction time to maintain pH stability. This approach was used to reduce local alkaline microenvironments and improve pH homogeneity throughout the flask.

Figure 4.4 reports the  $^1\text{H}$  NMR spectrum obtained under these updated conditions. Compared to the initial syntheses, the spectrum indicates a higher degree of methacrylation (~140%), consistent with a successful progression of the functionalization reaction.

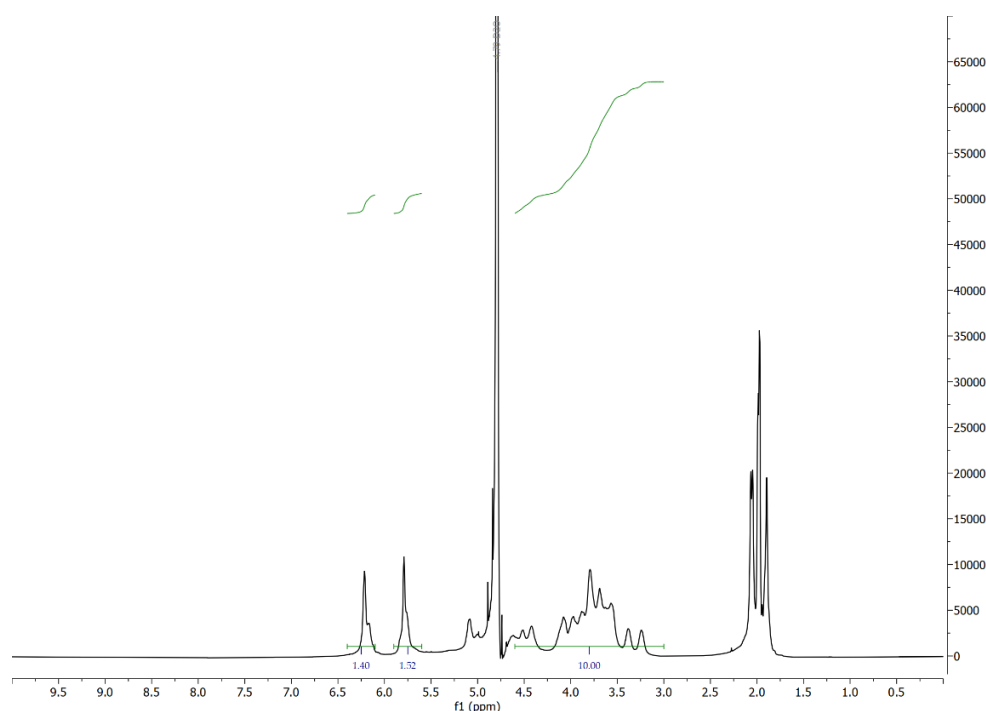


Figure 4.4:  $^1\text{H}$  NMR spectrum from Batch III (second synthesis protocol). Vinyl proton peaks at 5.6-6.1 ppm indicate successful methacrylation. Integration of vinyl peaks relative to HA backbone signals (3.0-4.6 ppm) yielded an apparent degree of methacrylation of ~140% (MestReNova), interpreted as an analytical overestimation consistent with near-complete methacrylation rather than true >100% substitution.

In this synthesis, as well as in several additional batches reported in Appendix A, the degree of methacrylation calculated by software integration exceeded 100%. Since values above 100% are not physically meaningful for HA, these results are attributed to limitations of quantitative integration in polymer  $^1\text{H}$  NMR rather than to the underlying chemistry. In particular, the broad and overlapping HA backbone resonances, combined with baseline distortions related to residual solvent signal, can lead to underestimation of the reference backbone integral. As a consequence, the vinyl

proton signals associated with methacrylate groups may be overestimated by manual integration.

In principle, values above 100% could suggest additional substitution beyond the primary hydroxyl target. However, under the mild pH conditions used in this synthesis, methacrylation is expected to occur predominantly at primary hydroxyl group of the *N*-acetyl-*D*-glucosamine unit, while extensive functionalization of secondary hydroxyl groups is unlikely [102]. Therefore, the >100% values are interpreted as analytical overestimation and should be considered indicative of near-complete methacrylation rather than absolute quantitative values.

Unfortunately, this functionalization result was not reproducible in subsequent syntheses. This lack of batch-to-batch reproducibility has already been identified as a major limitation in the literature on HA methacrylation [101]. Figure 4.5 reports the  $^1\text{H}$  NMR spectrum obtained under the same conditions, but it clearly shows a reduction in methacrylation of the HA backbone (as well as in spectra reported in Figure A.2 in the Appendix A).

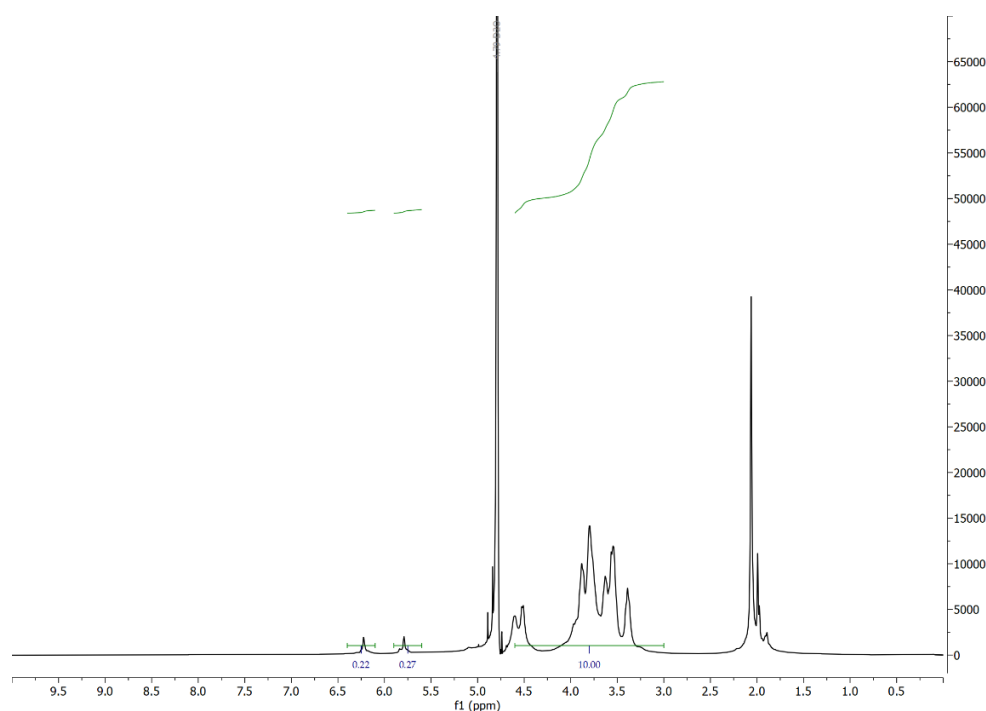


Figure 4.5:  $^1\text{H}$  NMR spectrum of Batch IV (second synthesis protocol). Reduced intensity of vinyl proton peaks (5.6-6.1 ppm) indicates a lower degree of methacrylation. Integration relative to the HA backbone (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~25% (MestReNova), highlighting limited batch-to-batch reproducibility under this protocol.

This reduced functionalization may be explained by two factors: an excessively large reaction volume caused by the addition of 0.1 M NaOH and therefore associated with

reduced effective collisions between reactants and a possible decrease in methacrylic anhydride reactivity during storage.

#### 4.1.2.1. Methacrylic anhydride degradation

To evaluate whether methacrylic anhydride degradation contributed to poor reproducibility of methacrylation degree,  $^1\text{H}$  NMR analysis was performed.

Figure 4.6 reports the  $^1\text{H}$  NMR spectrum of methacrylic anhydride dissolved in dimethyl sulfoxide  $d_6$  (deuterated DMSO). The spectrum displays the expected peaks of intact methacrylic anhydride: two vinyl proton signals in the olefinic region (around 5.7-6.2 ppm) and one methyl signal at approximately 1.9 ppm corresponding to the  $\text{CH}_3$  groups attached to the  $\text{C}=\text{C}$  units. The presence of these characteristic peaks is consistent with reference spectra reported by the manufacturer and available on SpectraBase. Therefore, methacrylic anhydride degradation was excluded as the primary cause of the inconsistent functionalization results.

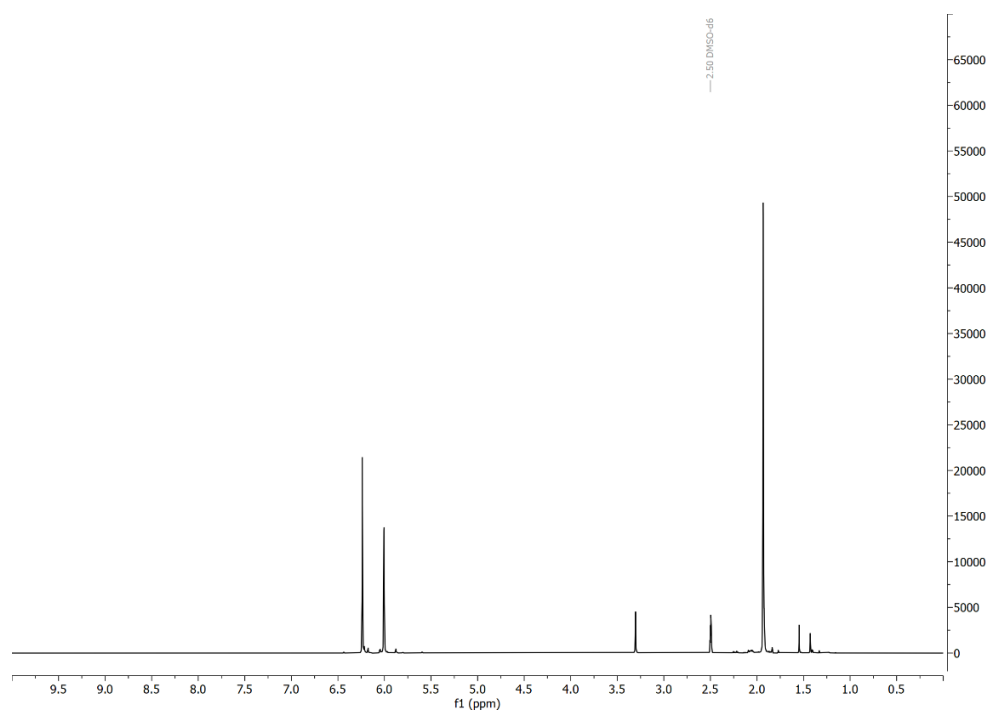


Figure 4.6:  $^1\text{H}$  NMR spectrum of methacrylic anhydride. The presence of the characteristic vinyl proton resonances (~5.7-6.2 ppm) and methyl resonance (~1.9 ppm) matches reference spectra for intact methacrylic anhydride, indicating no evident degradation under the storage conditions used.

#### 4.1.3. Third synthesis protocol

After confirming that methacrylic anhydride quality was not the limiting factor, the synthesis protocol was further adjusted. Specifically, 1 M NaOH was used throughout

the reaction to limit the increase in total reaction volume and methacrylic anhydride was added gradually (1 mL every 20-30 minutes) rather than in single addition. This stepwise addition was intended to improve reagent dispersion within the reaction mixture and to avoid local concentration gradients; a similar strategy has been associated with improved methacrylation degree and batch-to-batch reproducibility by Pérez-Lloret *et al.* [101]. The protocol reported in the Materials and methods (Section 3.1.1) corresponds to this final optimized workflow.

Figure 4.7 reports the  $^1\text{H}$  NMR spectrum obtained under these new reaction conditions and shows a higher degree of functionalization compared with earlier syntheses. In addition, the spectra reported in Appendix A (Batches VII-XII) show improved reproducibility across independent batches.

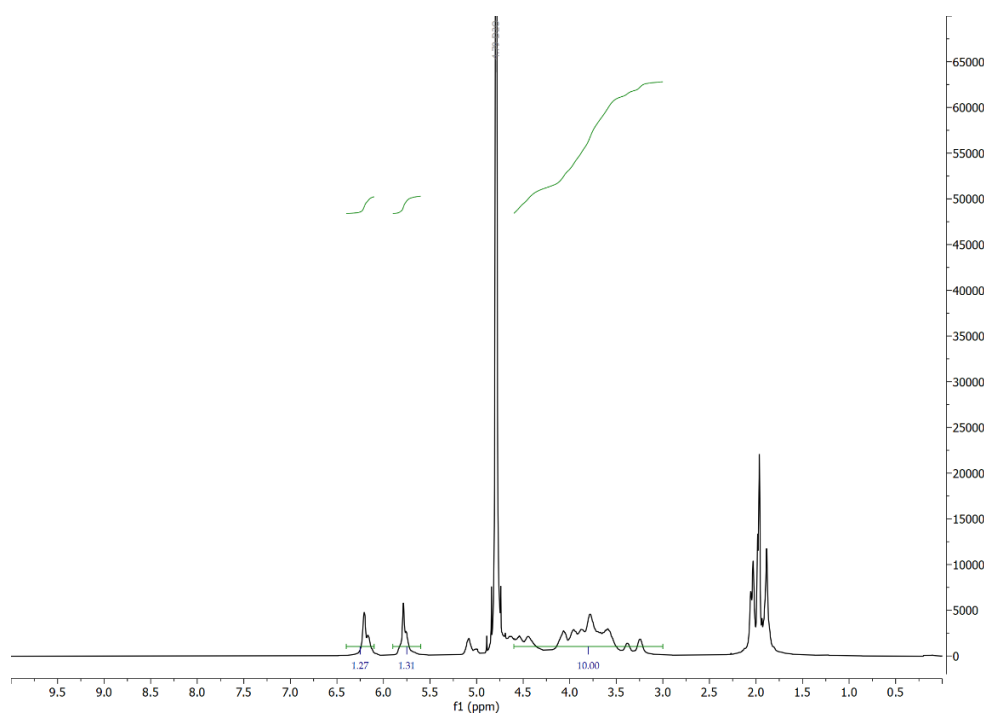


Figure 4.7:  $^1\text{H}$  NMR spectrum of Batch VI (third synthesis protocol). Clear vinyl proton peaks at 5.6-6.1 ppm indicate successful methacrylation of the HA backbone. Integration of vinyl peaks relative to the HA backbone resonances (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~130% (MestReNova), interpreted as near-complete functionalization given known integration limitations in polymer  $^1\text{H}$  NMR.

Overall, these protocol modifications resulted in consistently higher methacrylation and improved batch-to-batch reproducibility. The optimized procedure was therefore adopted to produce approximately 10 g of total MeHA, which was pooled to obtain a uniform starting material for subsequent hydrogel fabrication. Figure 4.8 shows the  $^1\text{H}$

NMR spectrum of the pooled MeHA batches, confirming a high degree of functionalization consistent with the target of near-complete substitution. Minor resonances around ~1.1-1.2 ppm may be consistent with residual ethanol introduced during sample handling, as described in Section 3.3.

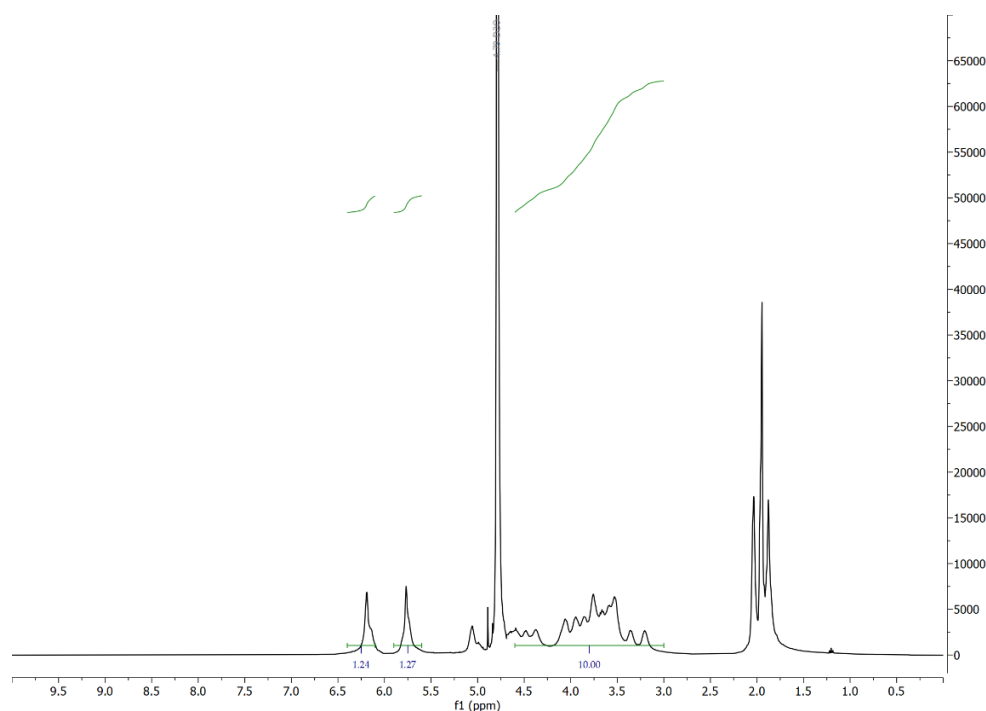


Figure 4.8: <sup>1</sup>H NMR spectrum of pooled MeHA. The persistence of vinyl proton peaks at 5.6-6.1 ppm confirms methacrylation in the pooled material. Integration relative to the HA backbone (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~125% (MestReNova), consistent with near-complete functionalization of the pooled MeHA.

## 4.2. Hydrogel fabrication and characterization

After synthesis, the different MeHA batches were pooled to minimize batch-to-batch variability in methacrylation degree and to obtain a starting material with more uniform functionalization for subsequent thiol-based crosslinking.

### 4.2.1. Bulk hydrogels

The hydrogel fabrication process began with bulk hydrogels formed by Michael-type addition using PEG(SH)<sub>2</sub> as linker. The thiol to methacrylate ratio was systematically varied to modulate crosslinking density and, consequently, hydrogel stiffness. In this design, the 1:1 condition was expected to produce the highest crosslink density and thus the highest stiffness, while the 1:10 condition was expected to yield the lowest effective crosslink density and the lowest stiffness. Figure 4.9 reports the rheological

measurements for all tested formulations. For each batch, at least three technical replicates were prepared and for each formulation at least four independent batches were produced and measured to obtain robust mean values.

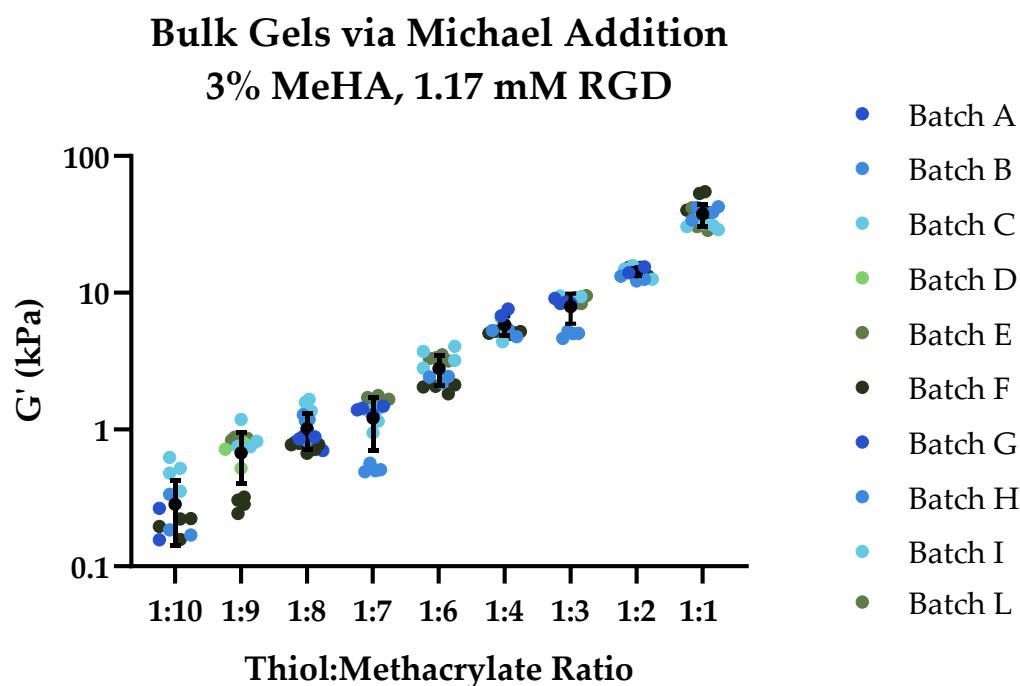


Figure 4.9: Bulk MeHA hydrogel stiffness as a function of thiol:methacrylate ratio. Storage modulus ( $G'$ , kPa) was measured by rheology for hydrogels prepared with thiol:methacrylate ratios from 1:10 to 1:1 (3% MeHA, 1.17 mM RGD). Colored points represent individual rheology measurements, color-coded by independent production batch ( $n = 4$  batches per formulation;  $n = 5$  for 1:8 formulation). Black points show the mean  $G'$  for each formulation, with error bars indicating the standard deviation calculated across all measurements pooled within that formulation.

The results show that Michael-type gelation enabled a broad stiffness window with mean storage modulus ( $G'$ ) values ranging from 0.28 kPa to 37.59 kPa (mean and standard deviation errors are reported in Table 2). As expected for chemically crosslinked HA-based networks, some variability was observed across individual measurements (including both within- and between-batch contributions), but the monotonic trend across formulations was consistent.

Moreover, the data confirm that the network mechanics can be tuned through thiol to methacrylate stoichiometry as mean stiffness values (black dots) increased with increasing PEG(SH)<sub>2</sub> content. This tunability was essential to identify formulations compatible with both physiological and pathological tissues.

Table 2: Summary stiffness values for bulk MeHA hydrogels across formulations. Mean  $\pm$  SD storage modulus ( $G'$ , kPa) for each thiol:methacrylate ratio, calculated across all rheology measurements pooled within the formulation (same dataset as Figure 4.9). Each formulation includes measurements from four independent batches (five for 1:8).

| $G'$ (kPa)                | 1:10 | 1:9  | 1:8  | 1:7  | 1:6  | 1:4  | 1:3  | 1:2   | 1:1   |
|---------------------------|------|------|------|------|------|------|------|-------|-------|
| <b>Mean</b>               | 0.28 | 0.68 | 1.01 | 1.21 | 2.79 | 5.83 | 7.91 | 14.26 | 37.59 |
| <b>Standard deviation</b> | 0.14 | 0.27 | 0.30 | 0.50 | 0.71 | 0.96 | 1.96 | 0.80  | 7.05  |

#### 4.2.2. Bulk hydrogels: *in situ* photo-stiffening

Methacrylate groups introduced along the HA backbone were exploited to implement a secondary crosslinking step based on photoinduced free radical polymerization. This strategy was used to generate dynamically stiffening hydrogels enabling temporal tuning of matrix mechanical properties rather than static conditions. In the context of wound healing and fibrosis, where extracellular matrix mechanics evolves over time, such dynamic control provides a more physiologically relevant *in vitro* system.

Previous studies have shown that cells respond differently to static versus time-varying mechanical environments. Using related hydrogel platforms, Guvendiren *et al.* and Caliri *et al.* demonstrated that dynamic stiffening alters cellular behavior compared with static stiffness conditions, including effects on mesenchymal stem cell responses and hepatic stellate cell activation [88], [89]. From these observations, the present work investigates dynamic stiffening conditions to identify mechanically relevant formulations for subsequent use in a 3D system.

Before testing dynamic stiffening conditions, it was necessary to define target stiffness ranges representative of healthy and pathological skin. Importantly, reported skin stiffness values vary substantially across studies because measured values depend on multiple factors including measurement technique, anatomical site, depth and layer analyzed and subject-specific variables such as age and gender. This variability is well documented in the skin biomechanics literature and must be considered when selecting *in vitro* mechanical targets [103], [104], [105].

From literature, Figure 4.10 shows AFM measurements of papillary and reticular human dermis reported by Wahlsten *et al.* Based on these data, a stiffness value of approximately 3 kPa was selected as a representative healthy reference for healthy dermis in this work [103]. For pathological conditions, fibrotic skin is generally reported as mechanically stiffer than healthy tissue as a result of an increased quantity of extracellular matrix, with ranges frequently extending into the tens of kPa (10-30 kPa) depending on model, tissue source and method of measurement.

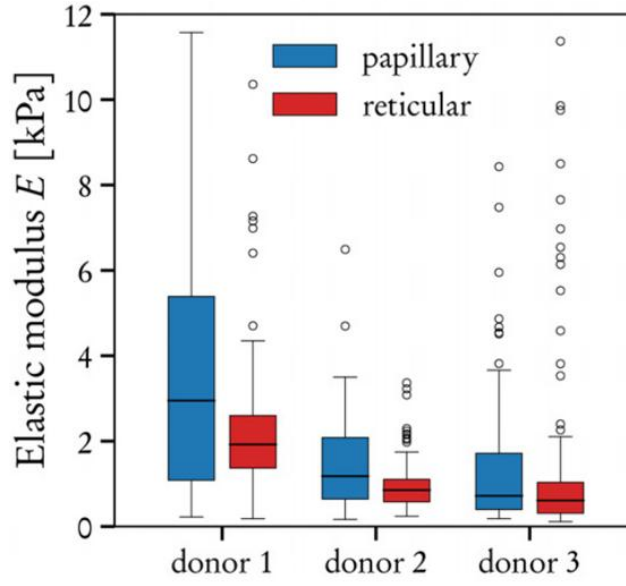


Figure 4.10: Elastic modulus ( $E$ ) of human dermis measured by AFM indentation for three donors, reported separately for papillary (blue) and reticular (red) dermis. Boxes indicate interquartile range with median line; whiskers extend to  $1.5\times$  the interquartile range. Adapted from [103].

At this stage, a methodological clarification is required. In the skin biomechanical literature, stiffness values are often reported as elastic modulus ( $E$ ), whereas rheological characterization in this work, as in hydrogel studies in general, provides the storage shear modulus ( $G'$ ). These parameters are not identical, but they are related through Poisson's ratio ( $\nu$ ) as shown in (4.1):

$$E = 2G(1 + \nu) \quad (4.1)$$

where  $E$  is the elastic modulus,  $G$  is the shear modulus and  $\nu$  is Poisson's ratio. Since most hydrogels are considered nearly incompressible ( $\nu \approx 0.45 - 0.5$ ), a common approximation is  $E \approx 3G$  [79], [106]. In this work, the shear modulus  $G$  was approximated using  $G'$ , because oscillatory measurements under the selected conditions consistently showed  $G' \gg G''$ .

Based on this approximation and on the stiffness values reported in Table 2, formulations 1:9, 1:8, 1:7 and also 1:6 were considered representative of physiological (soft) conditions, while 1:4, 1:3 and 1:2 were considered representative of pathological fibrotic (stiff) conditions. The rationale was to identify conditions that could be dynamically stiffened from a soft state toward a stiffer state, enabling investigation of cell responses to temporal mechanical changes rather than only static soft or static stiff environments.

Secondary crosslinking by free radical polymerization was first tested using 0.1% LAP to estimate the stiffening potential across formulations. Figure 4.11 reports  $G'$  values

measured before secondary crosslinking (static condition, non-filled dots) and after dynamic stiffening (filled dots). An increase in  $G'$  was observed for all tested formulations after light exposure, confirming effective secondary network formation.

Notably, the softer formulations 1:9 and 1:8 reached post stiffening  $G'$  values comparable to the static values of the stiffer 1:4 condition. This result indicated that these softer starting samples can be stiffened into a pathological-like stiffness through secondary crosslinking, supporting the feasibility of a dynamic *in vitro* model.

For 1:10 and 1:8, fewer post stiffening data points were available compared to pre stiffening (initial) data points because some samples were damaged during handling and reloading on the rheometer after PBS swelling.

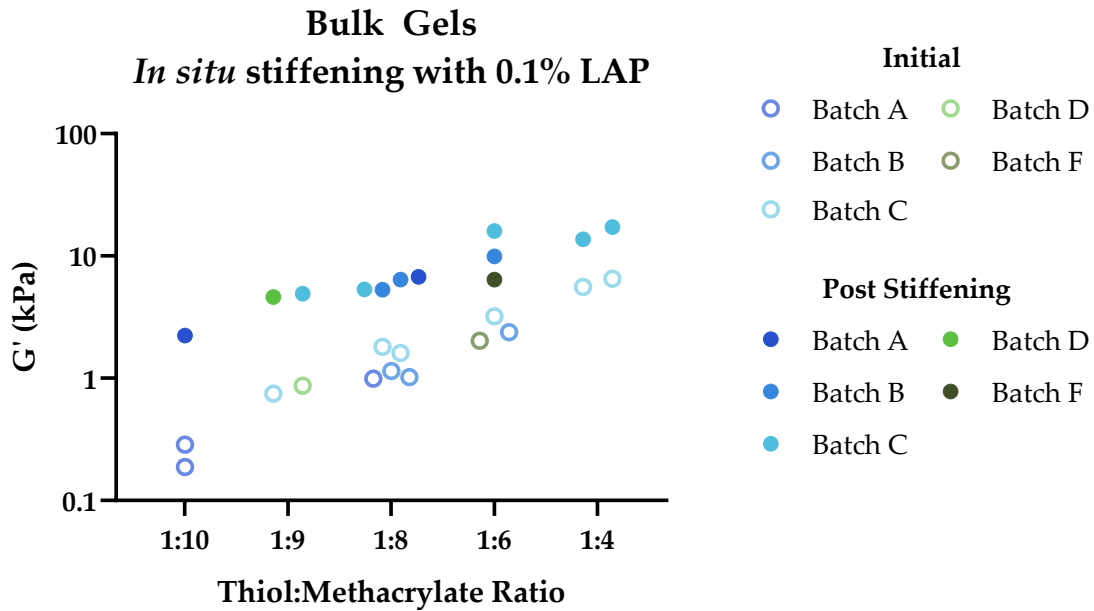


Figure 4.11: *In situ* stiffening of bulk MeHA hydrogels using 0.1% (w/v) LAP. Storage modulus ( $G'$ ) measured before irradiation (non-filled circles) and after photo-crosslinking (filled circles) for multiple thiol:methacrylate ratios. Colors denote independent production batches. Photo-crosslinking increased  $G'$  across formulations, demonstrating tunable stiffening via secondary network formation.

To further investigate secondary crosslinking under conditions more suitable for cell-related applications, hydrogels were also stiffened using 0.01% LAP concentration. In this study, 0.01% and 0.1% LAP correspond to approximately 0.34 mM and 3.4 mM respectively (considering  $MW_{LAP} = 294.21$  g/mol). Although higher LAP concentrations (up to 6.6 mM) have been reported as cytocompatible in comparable MeHA systems, reducing the photoinitiator concentration was expected to limit radical exposure and to slow the stiffening process [89]. This aspect is particularly relevant for the present

platform, where matrix stiffness is intended to evolve progressively, as occurs during wound healing and fibrosis, rather than as an instantaneous transition.

Figure 4.12 summarizes the rheological data for samples before and after photoinitiated stiffening with 0.01% LAP. In addition to the single stiffening strategy used for 0.1% LAP, a second approach was tested in which hydrogels underwent two consecutive photoinduced free radical polymerization steps, separated by two hours of swelling in PBS (darker filled dots). The objective was to determine whether a lower initiator concentration, combined with sequential crosslinking, could achieve final  $G'$  values comparable to those obtained with a single stiffening step at 0.1% LAP, while providing improved temporal control over stiffness evolution.

Notably, for the 1:9, 1:7 and 1:6 formulations, the double stiffening condition enabled a clear increase in  $G'$ , with final values approaching the stiffness range of the 1:4 condition similarly to what was observed in Figure 4.11. These results indicate that, at lower LAP concentration, a second crosslinking step was required to produce a sufficiently large stiffness increase; in addition, this strategy can be used to extend the stiffening timeline and produce a more gradual increase in stiffness over time.

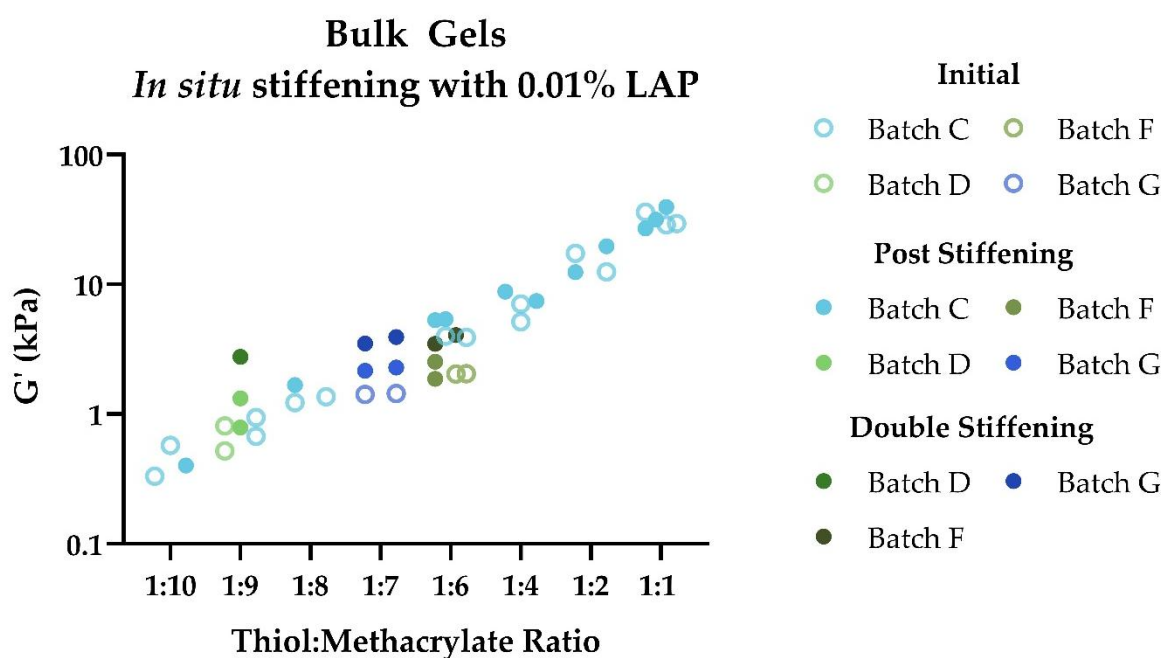


Figure 4.12: *In situ* stiffening of bulk MeHA hydrogels using 0.01% (w/v) LAP. Storage modulus ( $G'$ ) measured before irradiation (non-filled circles), after a single stiffening step (light filled circles) and after two sequential stiffening steps (dark filled circles). Colors denote independent batches. Sequential irradiation increased  $G'$  further compared to a single exposure, enabling more gradual stiffening trajectories at lower initiator concentration.

Although LAP induced stiffening in MeHA systems has been reported as cytocompatible by Caliri *et al.*, cytocompatibility remains condition dependent. Therefore, for the specific system and stiffening conditions used in this work (405 nm

light, 10 mW/cm<sup>2</sup>, 10 minutes exposure), viability should be evaluated directly under the same setup to confirm that the selected irradiation protocol and initiator concentration are compatible with subsequent cell experiments [89].

#### 4.2.2.1. Selection of formulations for static and dynamic stiffness conditions

To summarize the formulations more suitable for reproducing *in vitro* stiffness ranges relevant to wound healing and fibrosis, selected conditions from both 0.1% LAP and 0.01% LAP were compared to define candidate static and dynamic conditions for future cell experiments.

For 0.1% LAP (Figure 4.13), rheological results identified formulations 1:9 and 1:8 as soft conditions within a physiologically relevant range, while 1:4 represented a stiffer, pathologically relevant condition. Under this initiator concentration, secondary crosslinking produced a clear increase in  $G'$  across the tested formulations and dynamic transitions from soft starting conditions toward the 1:4 stiffness were achieved. These data support the use of 0.1% LAP as a robust stiffening conditions to generate distinct static and dynamic states.

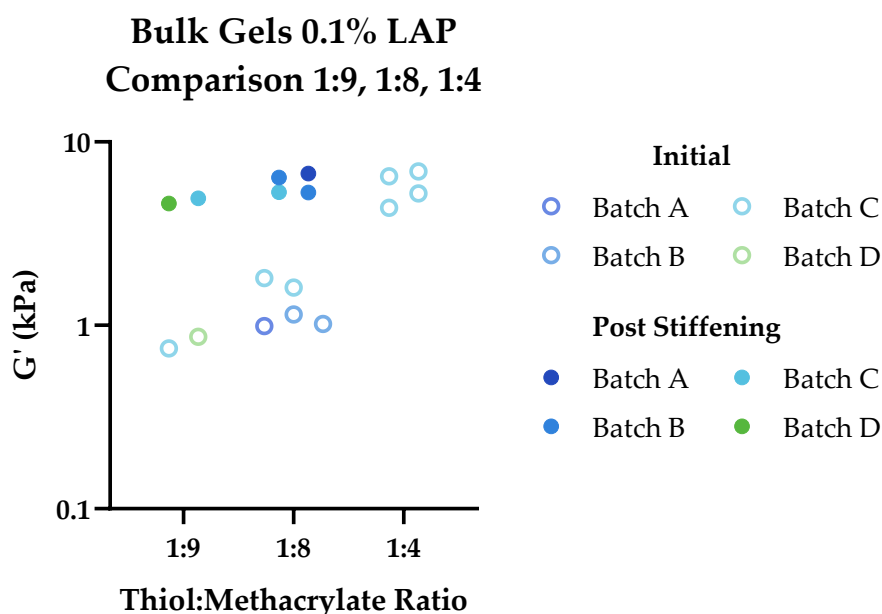


Figure 4.13: Comparison of selected bulk formulations (1:9, 1:8, 1:4) stiffened with 0.1% (w/v) LAP. Storage modulus ( $G'$ ) is shown before irradiation (non-filled circles) and after stiffening (filled circles). Colors denote independent batches. Softer starting formulations (1:9, 1:8) reached post-stiffening values approaching the static stiffness range of the stiffer 1:4 formulation.

For 0.01% LAP (Figure 4.14), the selected formulation set included 1:9, 1:8, 1:7 and 1:6 as viable soft conditions mimicking healthy skin, while 1:4 was selected as stiffer conditions mimicking fibrotic conditions. The lower initiator concentration still

enabled effective stiffening while producing a more gradual increase in final  $G'$  values compared with 0.1% LAP. Sequential irradiation further increased stiffness for the tested formulations and improved access to the target stiffer range from softer initial states.

Notably, double stiffening could not be completed for the 1:8 condition because samples ruptured during handling and reloading after swelling. Overall, the 0.01% LAP strategy is relevant when a slower mechanical evolution is desired while preserving the possibility of reaching pathologically relevant stiffness levels.

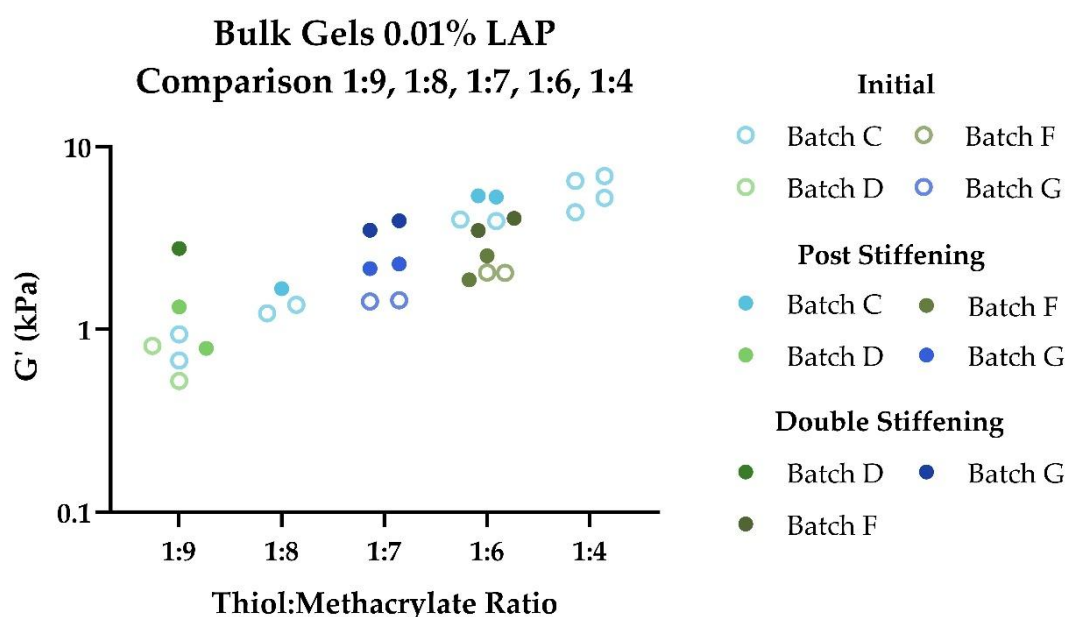


Figure 4.14: Comparison of selected bulk formulations (1:9, 1:8, 1:7, 1:6, 1:4) stiffened with 0.01% (w/v) LAP. Storage modulus ( $G'$ ) is shown before irradiation (non-filled circles), after a single stiffening step (light filled circles) and after double stiffening (dark filled circles). Colors denote independent batches. Lower initiator concentration produced smaller stiffness increases per exposure, while sequential irradiation improved access to the target stiffness range of the 1:4 formulation.

Overall, both strategies provide usable dynamic changes to mechanical environment that can be used with future cell experiments, with 0.1% LAP favouring one step stiffening and 0.01% LAP offering finer temporal control through sequential crosslinking.

### 4.2.3. Granular hydrogels

After preparing bulk hydrogel formulations and identifying conditions relevant to physiological and pathological stiffness ranges, the next step was to process these materials into granular hydrogels. This was required to enable loading into

microfluidic devices and to create a three-dimensional microenvironment for cell culture.

#### 4.2.3.1. First method

Figure 4.15 reports the rheological characterization of granular hydrogels prepared from different formulations and batches. In contrast to bulk hydrogels, the data did not show a clear increase in stiffness from softer to stiffer formulations (for examples, from 1:10 toward 1:1), as would be expected based on bulk hydrogels behavior.

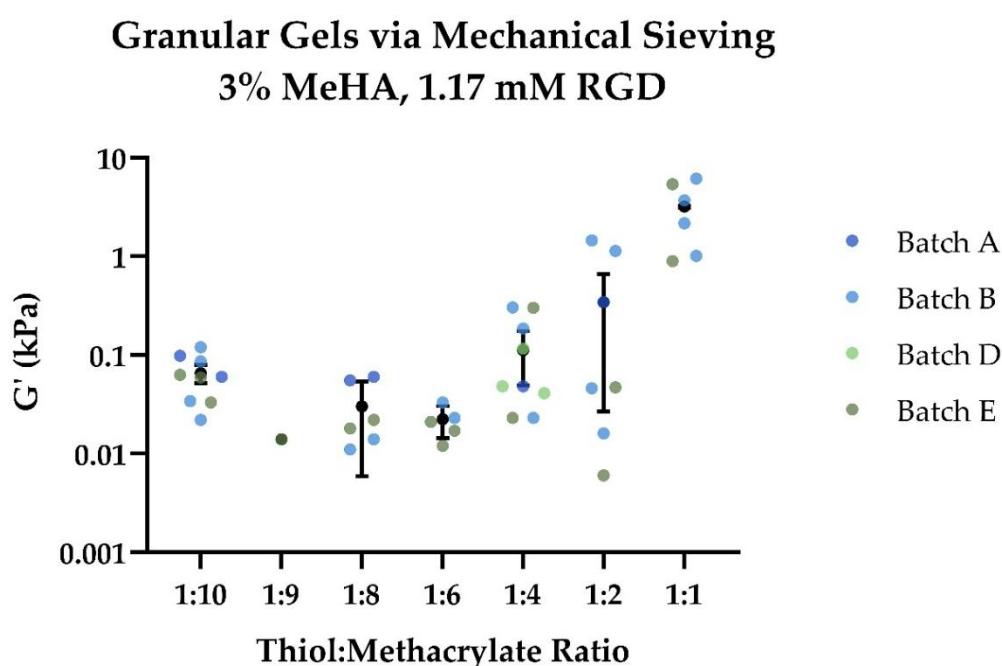


Figure 4.15: Rheological characterization of granular MeHA hydrogels prepared by mechanical sieving (first method). Storage modulus ( $G'$ ) values are shown for multiple thiol:methacrylate ratios; colors denote independent batches. Black points show the mean  $G'$  for each formulation with error bars indicating the standard deviation calculated across all measurements pooled within that formulation. Measurements show high dispersion and weak formulation dependence, consistent with sensitivity of granular mechanics to jamming state and residual liquid content during sample preparation/loading.

In general, measurements were highly variable within the same batch and even more across batches of the same formulation. A likely technical cause was the incomplete removal of the supernatant phase before sample loading on the rheometer, which may have altered the effective jamming state of the granular phase and introduced variability in measured  $G'$ .

For softer formulations, an additional contributing factor may have been insufficient swelling medium after sieving (500  $\mu\text{L}$  gel in 1 mL medium). Under these conditions,

hydration of the granular phase may have been not optimal, further reducing consistency between formulations.

#### 4.2.3.2. Second method

To address these limitations, two protocol modifications were introduced. First, the swelling condition was changed to 250  $\mu\text{L}$  gel in 1.75 mL medium. Second, sample handling before rheological measurement was refined to maximize removal of the liquid phase prior to loading, as described in Materials and Methods (Section 3.5.3.2).

Figure 4.16 reports the rheological results obtained with this refined protocol, including two to three independent batches per condition. Compared with the first method, the data show a clearer increase in stiffness with increasing PEG(SH)<sub>2</sub> content, consistent with the trend observed in bulk hydrogels. In addition, inter batch dispersion was reduced, indicating improved reproducibility of granular hydrogel characterization.

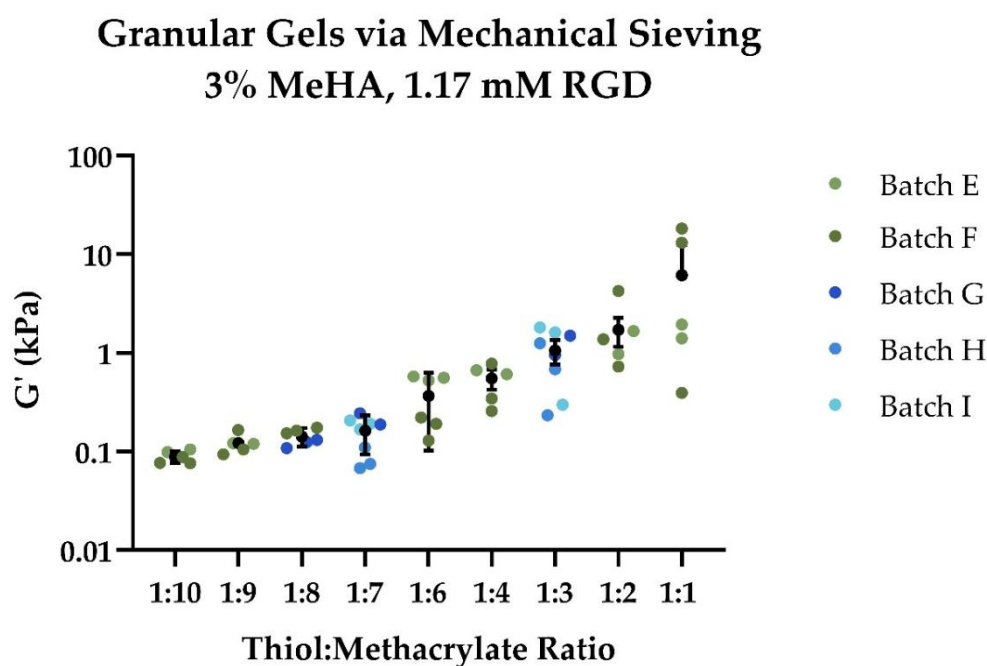


Figure 4.16: Rheological characterization of granular MeHA hydrogels using a refined preparation/loading method (second method). Storage modulus ( $G'$ ) values are shown for multiple thiol:methacrylate ratios; colors denote independent batches. Black points indicate the mean  $G'$  for each formulation with error bars showing  $\pm$  SD across all measurements pooled within that formulation. Compared to Figure 4.15, the refined workflow reduced variability and recovered a clearer stiffness trend with increasing PEG(SH)<sub>2</sub> content. Error bars reflect within-formulation variability and are visually amplified on the logarithmic y-axis for formulations with wider spread (notably 1:1).

These results indicate that granular hydrogel rheology is strongly influenced by sample handling and preparation before loading and that the optimized workflow

provides a more reliable basis for selecting formulations for subsequent microfluidic loading experiments.

### 4.3. Co-loading of granular hydrogels and cells in a microfluidic device

Following microfluidic device fabrication by soft lithography and plasma bonding to glass coverslips, granular MeHA hydrogels (jammed microgel assembly) were loaded into the confinement chamber to generate a stable three-dimensional matrix. The microfluidic device allowed the microgels to remain immobilized during media exchange, fixation and immunostaining, providing a practical workflow for imaging.

Neonatal human dermal fibroblasts (NHDF) were co-loaded with the granular hydrogels to assess short-term feasibility of maintaining cells within the confinement chamber and performing subsequent fluorescent staining and imaging. NHDF were cultured in the device for 24 hours before fixation. The objective at this stage was not to compare stiffness-dependent biological responses, but to verify co-loading of a cell-hydrogel suspension and the ability to visualize nuclei and cytoskeletal organization across multiple z-planes of the three-dimensional system.

For this experiment, the 1:2 formulation was selected because it enabled robust loading and retention within the confinement chamber, providing a reliable condition to evaluate staining and imaging in the device.

Figure 4.17 shows representative brightfield images after loading, indicating a densely packed granular network along the length of the chamber. Localized cell aggregates are visible in some regions (notably toward the bottom of the chamber), which may be attributed to incomplete homogenization of the cell-gel mixture prior to loading.

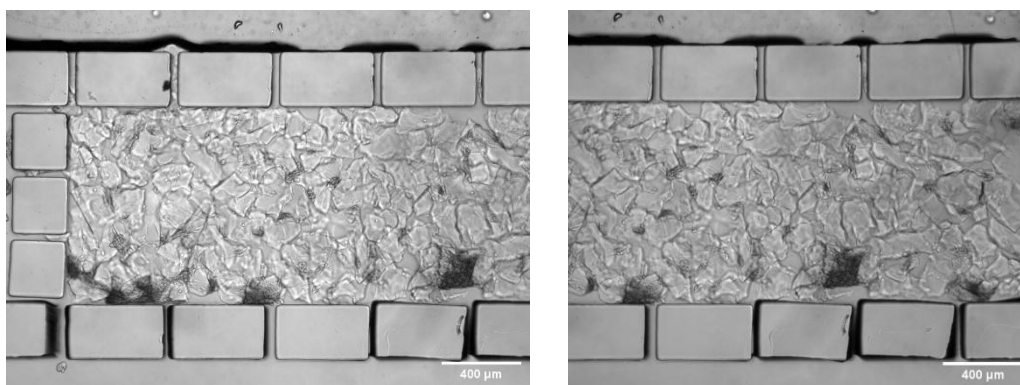


Figure 4.17: Brightfield images of the microfluidic confinement chamber after co-loading NHDF with a jammed granular MeHA hydrogel (1:2 formulation). The chamber is uniformly filled with a densely packed granular matrix retained by the pillar array, providing a stable three-dimensional scaffold for subsequent culture, fixation and immunostaining. Scale bar: 400  $\mu\text{m}$ .

After confirming complete jamming of the confinement chamber, NHDF were fixed and stained to visualize nuclei (DAPI, blue) and F-actin (phalloidin, magenta) within the three-dimensional granular network.

Figure 4.18 shows representative brightfield images and corresponding epifluorescence z-stacks visualized as maximum intensity projection (MIPs) from two locations within the confinement chamber (two columns). Z-stack imaging followed by MIP confirmed the presence of nuclei (DAPI) and F-actin (phalloidin) signal throughout the thickness of the system.

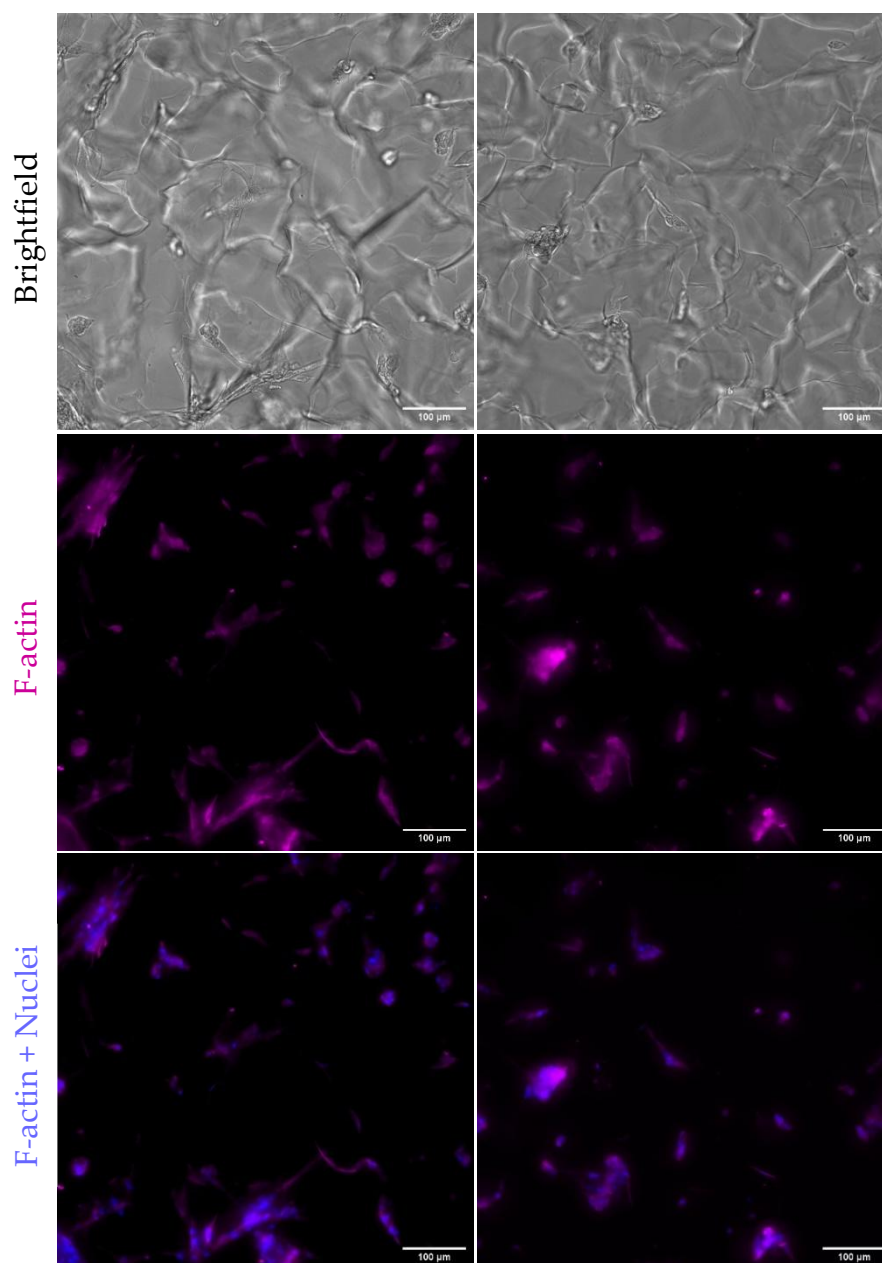


Figure 4.18: Widefield fluorescence imaging (20× objective) of NHDF within the granular MeHA matrix inside the microfluidic confinement chamber. Brightfield (top), F-actin (phalloidin, magenta; middle) and merged F-actin + nuclei (DAPI, blue; bottom) are shown for two representative regions. Images are maximum-intensity projections from z-stacks (left: 134 µm; right: 161 µm).

Qualitatively, the phalloidin signal exhibited heterogeneous patterns across both locations. In several regions, F-actin appeared concentrated in perinuclear areas, which is consistent with a rounded morphology and limited spreading. In other regions (for example in the lower part of the left location), F-actin appeared more distributed and elongated, suggesting local cytoskeletal organization and cell spreading within the three-dimensional granular matrix. Notably, this qualitative observation is consistent with the advantage of having a macroporous granular matrix in which cells reside within the interstitial void space, whereas in conventional nanoporous hydrogels cells are embedded within a dense polymer mesh that can restrict spreading [93].

Overall, these results demonstrate that the microfluidic device enables stable immobilization and retention of the granular network during fixation and immunostaining and supports fluorescence imaging throughout the three-dimensional chamber. As a next step, the mechanically and biologically relevant formulations established in this thesis will be used to compare cell responses across static soft and stiff conditions and after *in situ* stiffening within the microfluidic device.

#### 4.4. Monocytes isolation from whole blood

In parallel with the development of the hydrogel platform, preliminary work was carried out to establish the basis for future incorporation of human macrophages into the system. Although macrophage integration experiments could not be completed within the timeframe of this thesis, the monocyte isolation and flow cytometry phenotyping workflow was established as a necessary foundation for subsequent differentiation and integration. The long-term objective could be to include macrophages either within the same confinement chamber or in a dedicated multi-chamber microfluidic system to study immune cell recruitment and migration in response to fibroblast-derived cues.

To obtain monocytes, peripheral blood mononuclear cells (PBMCs) were first isolated from human blood using Ficoll-Paque density gradient separation. Monocytes were further isolated via negative immunomagnetic selection, which was chosen to maximize yield while minimizing potential cell phenotype alteration, as discussed by Nielsen *et al.* [107].

Flow cytometry was used to confirm successful monocyte isolation from whole blood. Events were first gated on the main cell population based on forward and side scatter, followed by singlet selection (FSC-A vs FSC-H) and exclusion of LIVE/DEAD-positive events (LIVE/DEAD Violet), yielding a live singlet population (Figure 4.19).

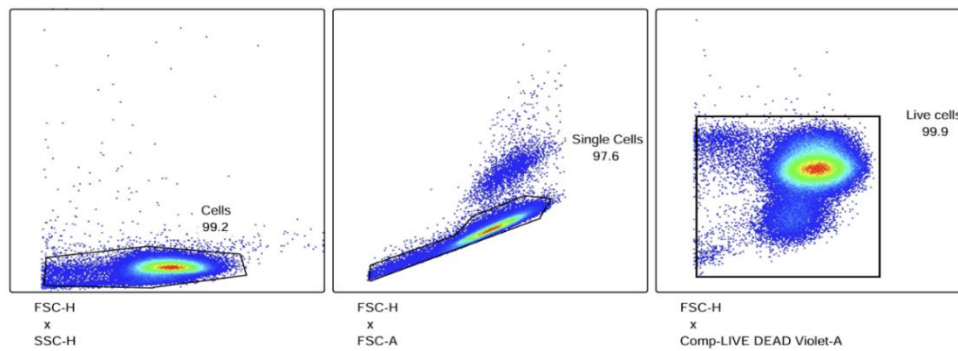


Figure 4.19: Flow cytometry gating strategy used to quantify monocyte isolation purity. Events were first gated on the main leukocyte population by forward/side scatter (FSC-H vs SSC-H), followed by singlet selection (FSC-H vs FSC-A) and exclusion of non-viable events using LIVE/DEAD Violet, yielding the live singlet population used for downstream CD14/CD16 phenotyping.

CD14 and CD16 positivity thresholds were defined using single stained compensation bead controls and kept constant across samples to ensure consistent quadrant placement (Figure 4.20). Within the live singlets cells, the majority of events (89.6%) were CD14<sup>+</sup>/CD16<sup>-</sup>, consistent with a predominantly classical monocyte population. A smaller CD14<sup>-</sup>/CD16<sup>-</sup> fraction (10.3%) remained, while CD14<sup>+</sup>/CD16<sup>+</sup> and CD14<sup>-</sup>/CD16<sup>+</sup> subsets were negligible, indicating minimal contaminations under these isolation conditions.

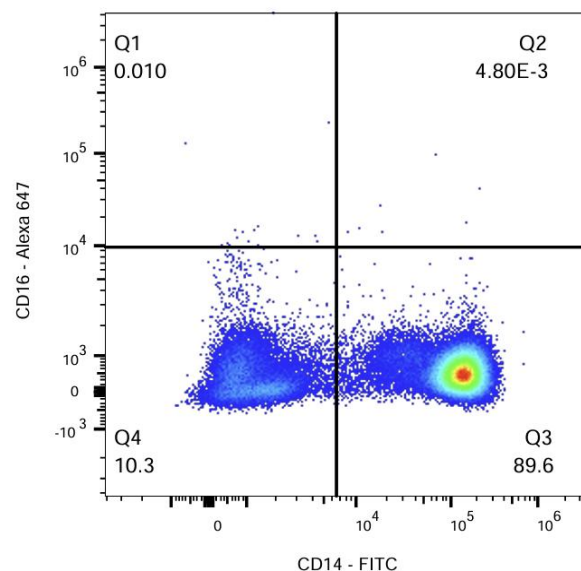


Figure 4.20: Flow cytometry CD14/CD16 phenotype of isolated primary monocytes after negative immunomagnetic enrichment. Quadrants were set using single-stained compensation bead controls and applied consistently across samples. Within the live singlet gate, the dominant population was CD14<sup>+</sup>/CD16<sup>-</sup> (classical monocytes, 89.6%), with a smaller CD14<sup>-</sup>/CD16<sup>-</sup> fraction (10.3%); CD16<sup>+</sup> subsets were negligible.

To further validate the phenotype characterization, the CD14/CD16 distribution obtained after isolation was compared with a representative monocyte profile reported in literature (Figure 4.21) from Nielsen *et al.* [107]. The two plots show a comparable overall distribution pattern, with the dominant population (~90%) located in the CD14<sup>+</sup>/CD16<sup>-</sup> region and a smaller fraction extending toward the CD14<sup>-</sup>/CD16<sup>-</sup> region. In addition, the quadrant distribution is in the same order of magnitude, supporting that the isolated cells display a phenotype consistent with primary human monocytes.

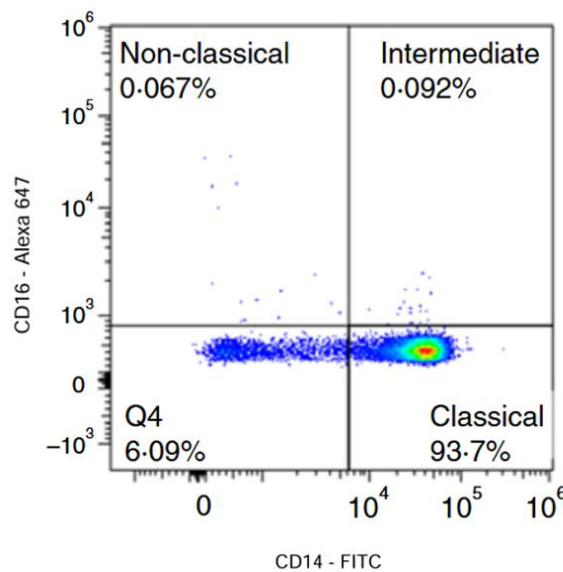


Figure 4.21: Representative CD14/CD16 monocyte subset distribution reported by Nielsen *et al.* The plot illustrates the typical predominance of classical CD14<sup>+</sup>/CD16<sup>-</sup> monocytes with smaller non-classical/intermediate fractions and is shown here as a reference for comparison with the phenotype obtained in this work (Figure 4.20). Adapted from [107].

The isolation procedure was subsequently repeated in additional independent experiments and flow cytometry phenotype characterization results were consistently reproducible (Appendix BB.1). These results indicate that the workflow provides a reliable technical basis for isolating monocytes and confirming their phenotype prior to their use in downstream applications.

Having confirmed the consistency and efficacy of the isolation protocol, the next step would be monocyte differentiation into macrophages using macrophage colony stimulating factor (M-CSF), a key regulator of monocyte to macrophage differentiation and maturation [107], [108]. This would enable integration of a primary immune component into the platform and support future studies investigating immune-stromal interactions in a mechanically tunable three-dimensional microenvironment.

## 5 Conclusions and future developments

This work established a reproducible photo-tunable three-dimensional hydrogel platform based on methacrylated hyaluronic acid (MeHA) to study mechanobiology in the context of skin wound healing and fibrosis. First, hyaluronic acid was successfully functionalized and a robust synthesis protocol was developed to obtain consistent degrees of methacrylation across multiple batches, as confirmed by  $^1\text{H}$  NMR. This provided a reliable starting material that enabled fabrication of reproducible hydrogel formulations across batches.

Using established chemistries, bulk MeHA hydrogels were fabricated and granular hydrogels were obtained by mechanical fragmentation [79], [99]. These steps enabled the production of well-characterized materials with tunable mechanical properties and supported the identification of formulations spanning stiffness ranges relevant to physiological wound healing and pathological fibrosis. A key outcome of the platform is the ability to expose cells to distinct mechanical histories, including static compliant matrices, static stiff matrices and dynamically stiffened matrices that better reflect the evolving mechanical environment of wound healing and fibrosis. Dynamic stiffening was achieved through sequential photo-crosslinking and the stiffening kinetics were tuned by varying photoinitiator concentration, with higher concentrations enabling faster stiffening and lower concentrations a more gradual increase.

To enable cell-based studies, PDMS microfluidic devices were fabricated and the granular matrix was loaded into the confinement chamber, enabling immobilization of the network during immunofluorescence staining and fluorescence imaging. Biocompatibility with primary dermal fibroblasts was observed and fibroblasts exhibited cell spreading and organized F-actin morphologies across multiple z-planes within the granular matrix. Notably, this behavior is consistent with the advantage of macroporous hydrogel networks in which cells reside in interstitial void space, whereas spreading can be limited in dense nanoporous hydrogels where cells are embedded within a restrictive polymer mesh.

Before systematic cell-based mechanobiology experiments, a limited set of validation steps remains important. First, cytocompatibility should be assessed under the specific photo-stiffening parameters used (photoinitiator concentration, light dose and exposure time). Second, because cellular responses may depend on both local mechanics and network behavior, the relationship between individual microgel

stiffness and the mechanics of the jammed granular assembly should be clarified. In the current workflow, hydrogel formulations were selected based on rheological characterization of bulk gels; future studies should compare whether cell phenotypes align more strongly with the stiffness of individual microgels or with the apparent mechanics of the packed granular network. Experimentally, this could be addressed by measuring and reporting both microgel-scale mechanics and the effective mechanics of the assembled granular material; then, by evaluating whether dermis-relevant mechanical targets produce cell responses consistent with known *in vivo* trends.

To further characterize the hydrogel architecture within the microfluidic setup and account for potential confinement effects that could alter cytoskeletal organization and activation state, fluorescent dextran (FITC-dextran) can be used to label the aqueous phase and image the fluid void space between microgels. This approach enables quantification of void fraction and pore size distribution and allows correlation of local microstructure with cell behavior within the channels. Importantly, repeating this analysis before and after dynamic stiffening would clarify whether stiffness changes are accompanied by changes in void space (and therefore confinement), helping to decouple mechanical stimulation from geometric constraints in downstream cellular mechanobiological responses [109], [110].

The next biological objective is to determine how dynamic increases in stiffness regulate fibroblast activation state over time. A working hypothesis is that *in situ* stiffening will increase cytoskeletal tension and adhesion maturation, promoting a transition toward a myofibroblast-like phenotype, particularly when combined with pro-fibrotic biochemical cues such as TGF- $\beta$ , which are present in wound-healing microenvironments. These temporal effects can be tested by comparing static soft, static stiff and dynamically stiffened conditions while varying the timing of stiffening relative to cell seeding and by quantifying responses at defined time points. Readouts can include early mechanotransduction responses and later functional outputs such as collagen I deposition, fibronectin fibrillogenesis and  $\alpha$ -SMA incorporation into stress fibers [44]. If activation is observed, mechanistic studies can extend beyond candidate pathways by applying transcriptomic profiling (RNA-sequencing) to identify programs associated with dynamic stiffening and to distinguish stiffness-driven responses from soluble factor-driven effects.

Notably, the platform achieves three-dimensional culture without relying on collagen as the base polymer, which is frequently used in wound-healing and fibrosis models. This is advantageous for downstream phenotyping because collagen-rich scaffolds can introduce background signal and complicate interpretation of newly deposited matrix. In contrast, a non-collagen MeHA matrix allows collagen I secretion to be interpreted more directly as a cell-derived output of fibroblast activation and ECM remodeling.

Beyond fibroblast-only studies, the platform can be expanded to better capture immune-stromal interactions by incorporating primary immune cells. The monocyte isolation protocol was demonstrated to be robust and to yield a high fraction of viable primary monocytes, providing a technical basis to increase model complexity and move closer to human biology. Primary monocytes could be used in two directions: studying monocyte migration and infiltration driven by fibroblast-derived chemokines under mechanically defined conditions or differentiating monocytes into macrophages and investigating macrophage-fibroblast crosstalk in three-dimensional matrices with controlled and evolving stiffness. This second direction is particularly relevant because macrophage states strongly influence fibroblast activation and fibrosis progression, while fibroblast-driven matrix remodeling can in turn shape macrophage phenotype [111].

In this thesis, dermal mechanics were used to identify physiologically relevant stiffness targets and dermal fibroblasts were used as a practical primary cell source; however, the same platform could be adapted to other fibrotic tissues by redefining the target stiffness window and substituting tissue-specific stromal and immune cell types.

From a translational perspective, understanding how mechanical cues shape fibroblast activation and macrophage polarization could inform strategies to reduce pathological scarring and fibrosis. A realistic translational path would be based on the identification of mechanistically grounded targets and design rules (for example, stiffness ranges and temporal windows that bias macrophages toward pro-resolving states and limit persistent myofibroblast activation). These insights could guide the development of advanced wound dressings or local biomaterials that modulate the inflammatory and mechanical microenvironment to promote resolution rather than chronic inflammation and scarring. Conversely, identifying signaling pathways associated with pro-fibrotic macrophage states could support the design of inhibitory strategies aimed at preventing sustained activation loops that reinforce fibrosis.



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# A Appendix A

## A.1. $^1\text{H}$ NMR spectra

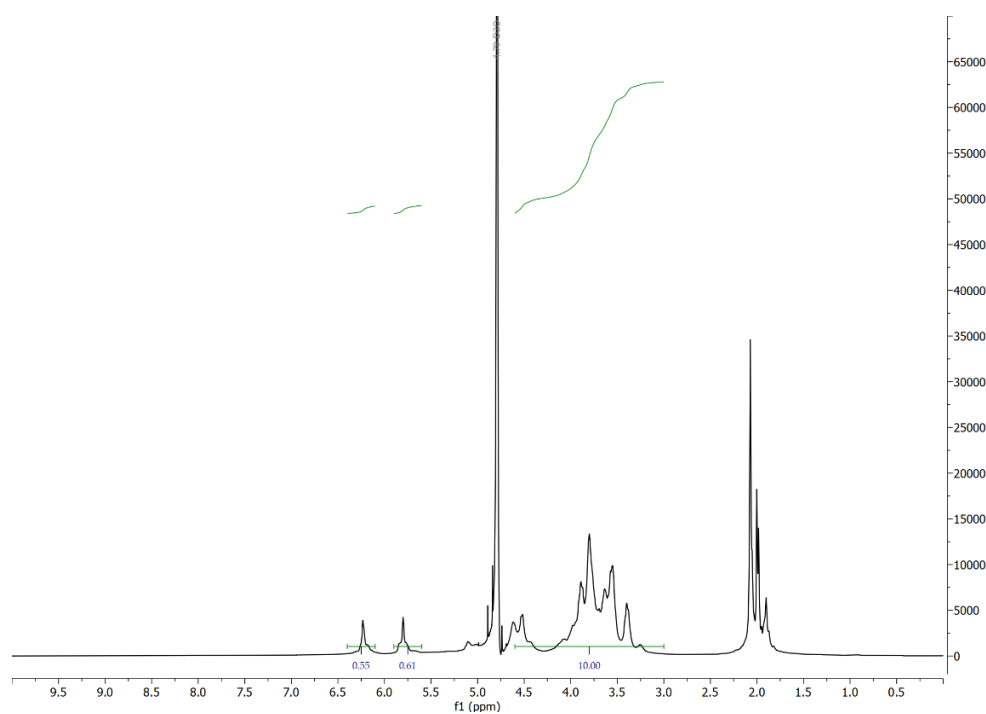


Figure A.1:  $^1\text{H}$  NMR spectrum of Batch II. Vinyl proton peaks at 5.6-6.1 ppm confirm methacrylate incorporation on the HA backbone. Integration of vinyl peaks relative to the HA backbone region (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~50% (MestReNova).

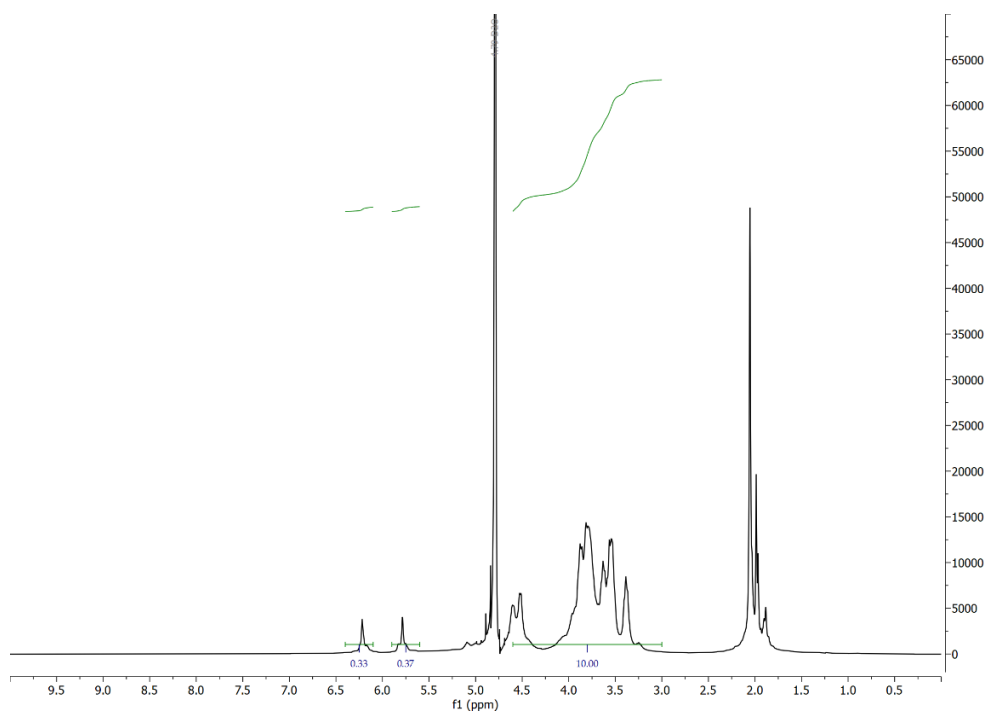


Figure A.2:  $^1\text{H}$  NMR spectrum of Batch V. Reduced vinyl peak intensity at 5.6-6.1 ppm indicates a lower degree of methacrylation. Integration relative to the HA backbone (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~35% (MestReNova).

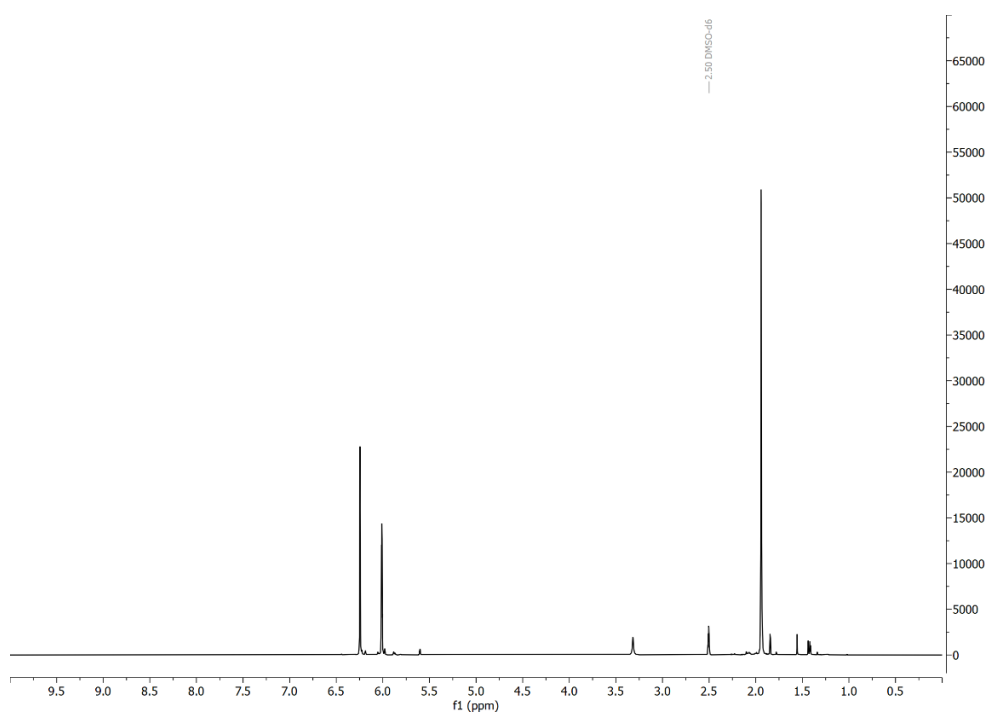


Figure A.3:  $^1\text{H}$  NMR spectrum of methacrylic anhydride (in  $\text{DMSO-d}_6$ ). Characteristic vinyl proton peaks in the olefinic region (5.7-6.2 ppm) and a methyl signal at ~1.9 ppm are consistent with intact methacrylic anhydride.

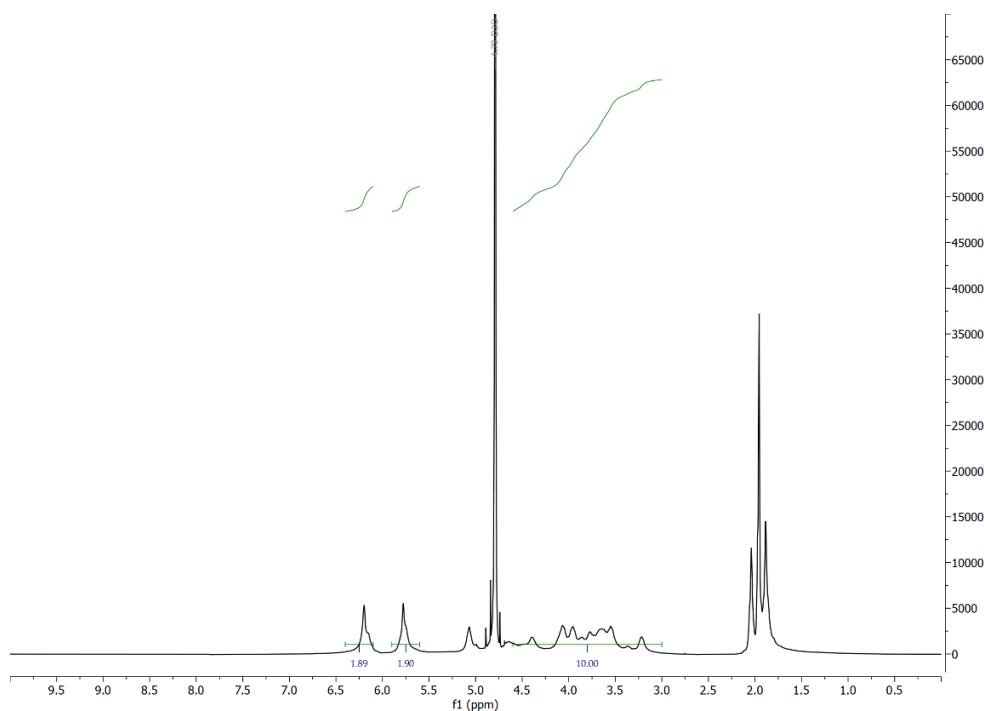


Figure A.4:  $^1\text{H}$  NMR spectrum of Batch VII. Pronounced vinyl peaks at 5.6-6.1 ppm indicate high methacrylation. Integration relative to the HA backbone region (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~190% (MestReNova), interpreted as an analytical overestimation consistent with near-complete methacrylation rather than true >100% substitution.

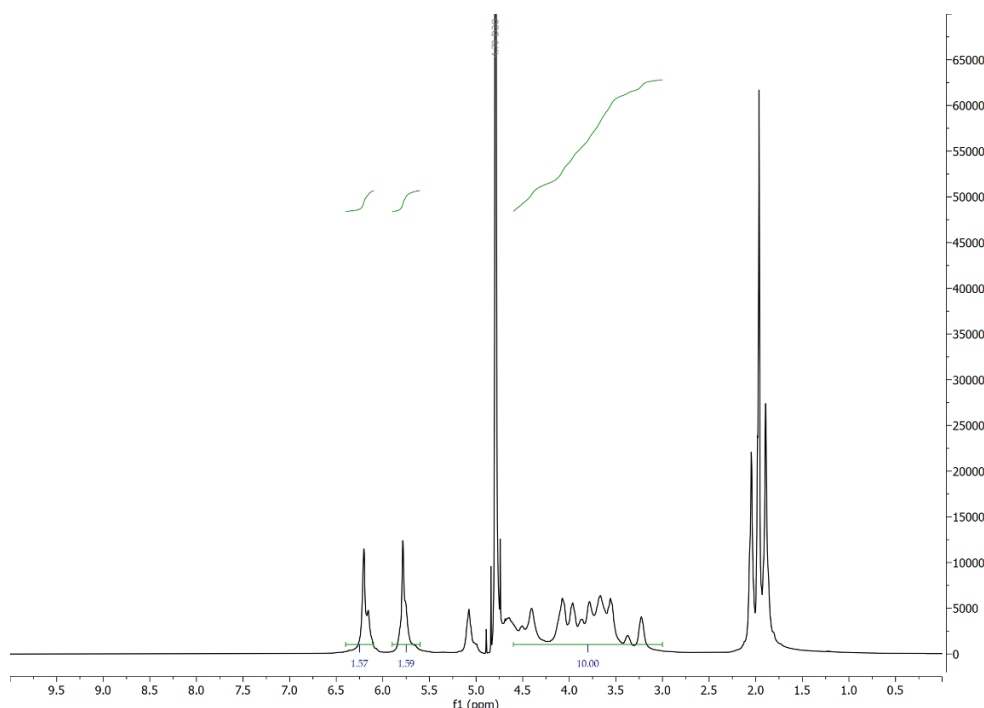


Figure A.5:  $^1\text{H}$  NMR spectrum of Batch VIII. Vinyl proton peaks at 5.6-6.1 ppm indicate high methacrylation. Integration relative to the HA backbone (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~160% (MestReNova), interpreted as near-complete functionalization with integration overestimation.

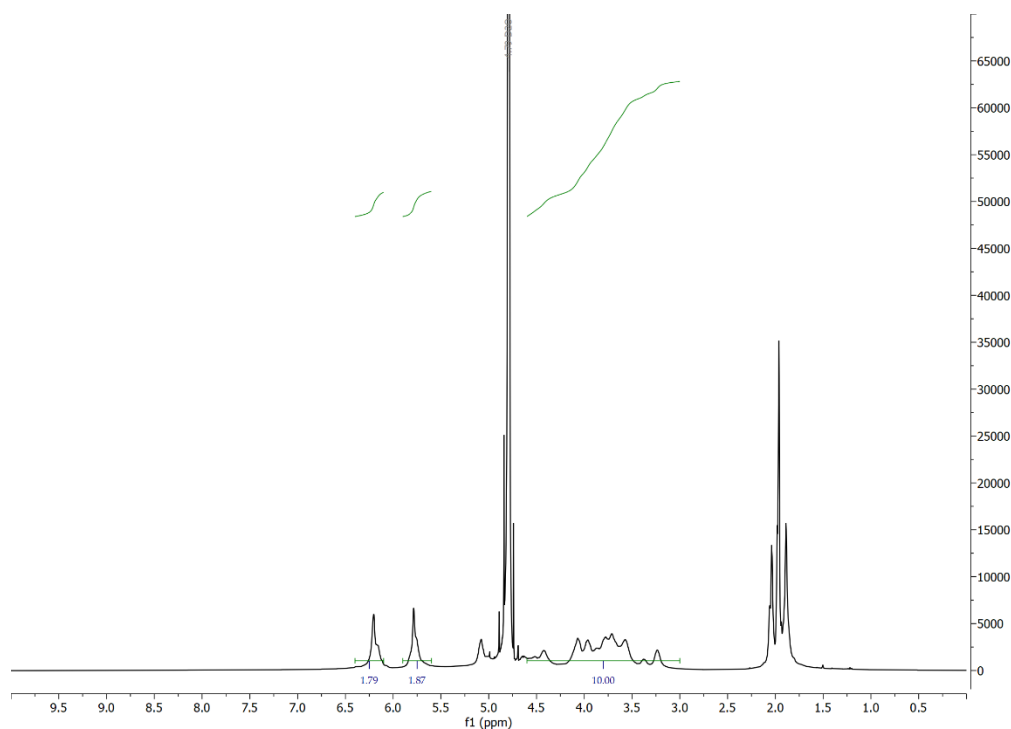


Figure A.7:  $^1\text{H}$  NMR spectrum of Batch IX. High-intensity vinyl proton peaks at 5.6-6.1 ppm indicate high methacrylation. Integration relative to the HA backbone (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~185% (MestReNova), interpreted as near-complete functionalization with integration overestimation.

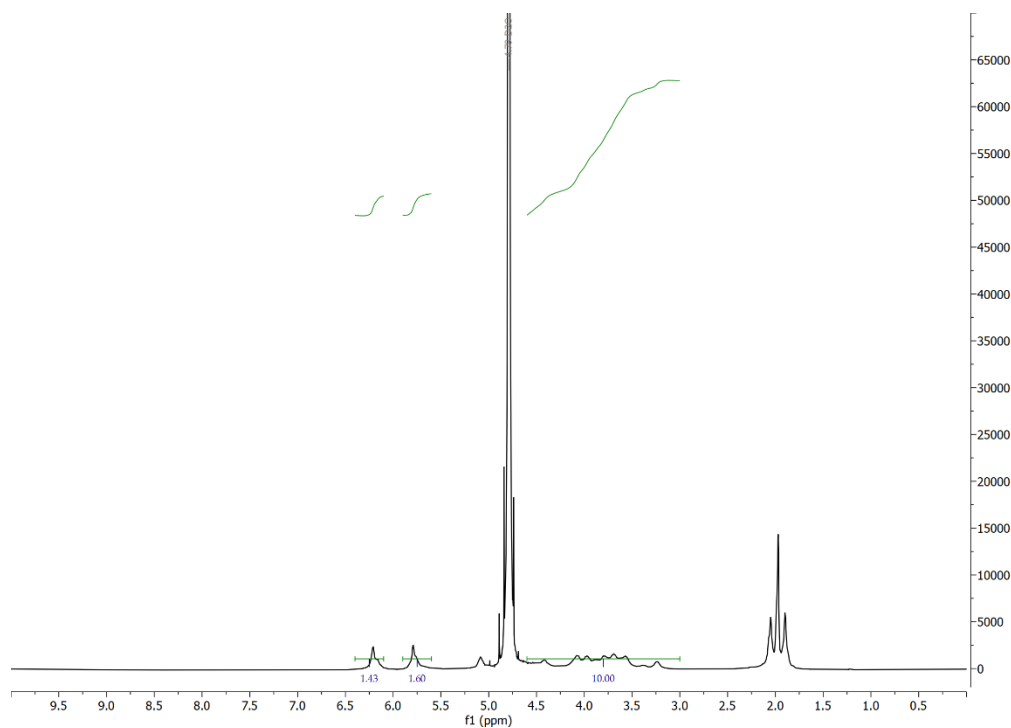


Figure A.6:  $^1\text{H}$  NMR spectrum of Batch X. Vinyl proton peaks at 5.6-6.1 ppm confirm methacrylation. Integration relative to the HA backbone (3.0-4.6 ppm) yielded an estimated degree of methacrylation of ~150% (MestReNova), interpreted as near-complete functionalization with integration overestimation.

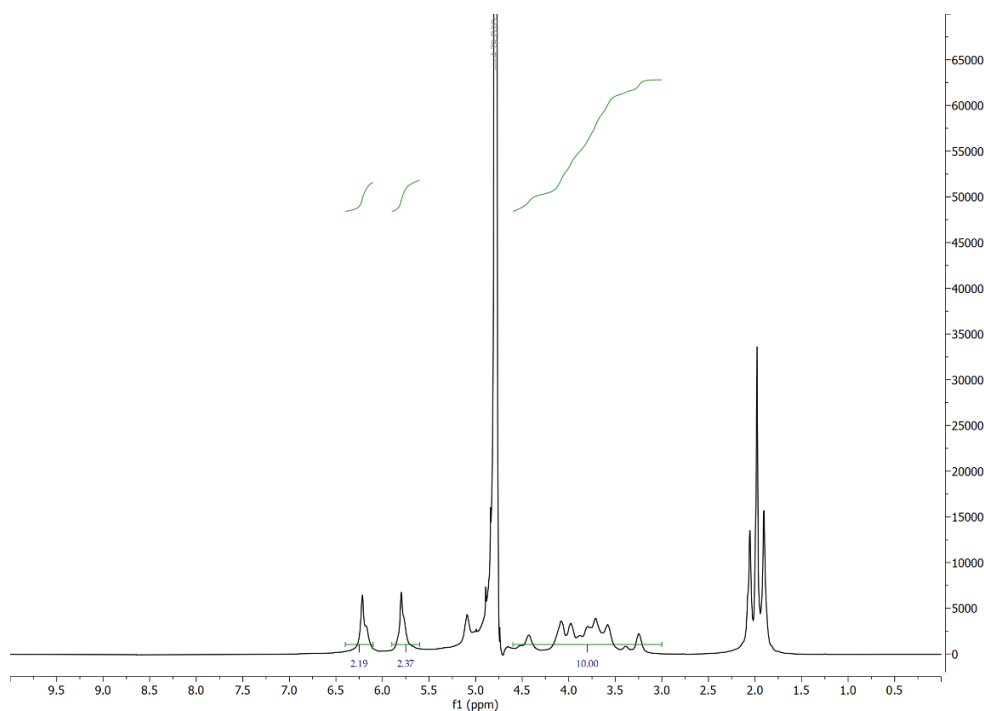


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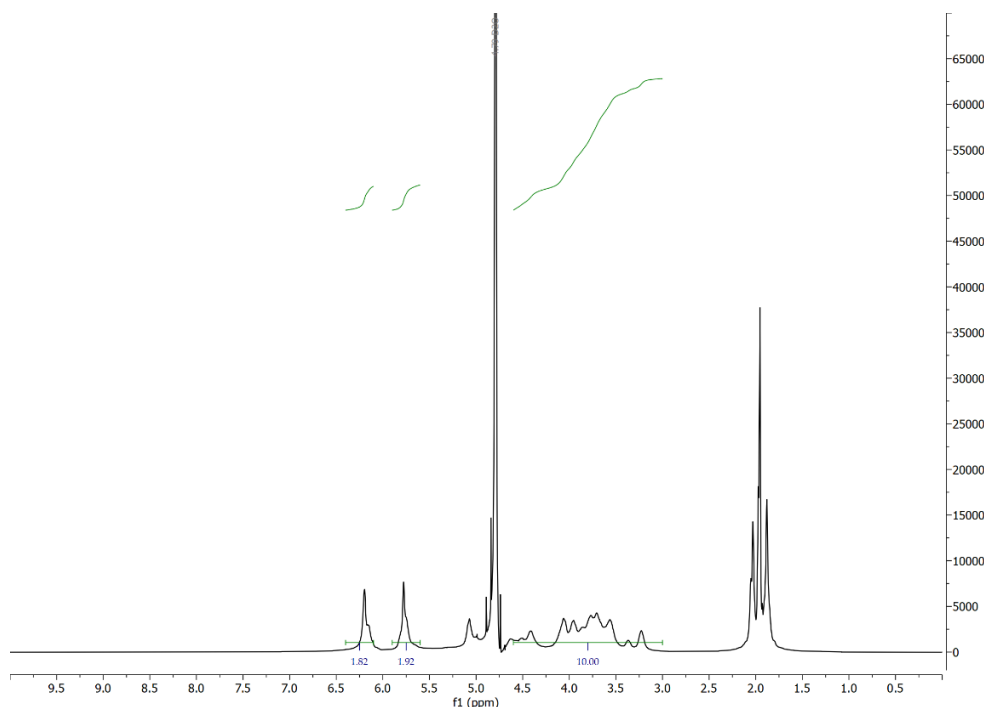


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## B Appendix B

### B.1. Flow cytometry (final CD14/CD16 readout)

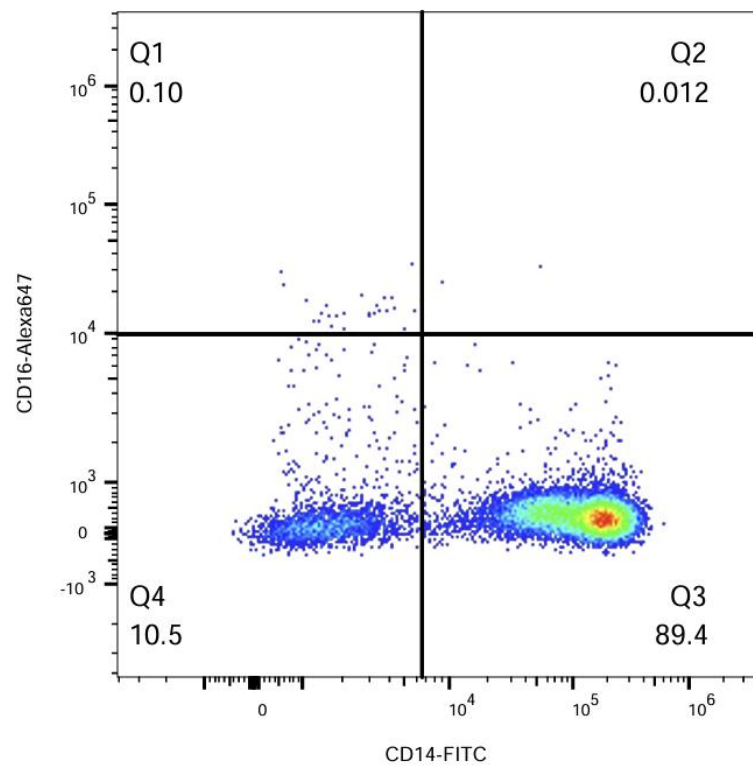


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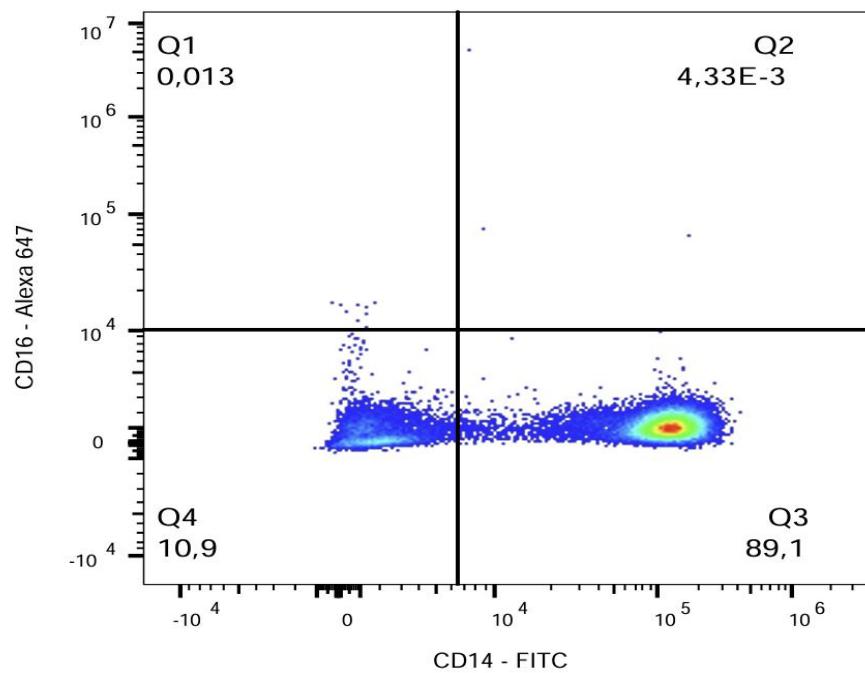


Figure B.2: Flow cytometry CD14 (FITC) vs CD16 (Alexa 647) profile of monocytes after the third isolation. Quadrant gates were applied as in Figure 4.20, showing a predominantly classical monocyte population ( $CD14^+/CD16^-$ ) with a smaller  $CD14^-/CD16^-$  fraction.

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## List of abbreviations

|                                |                                         |
|--------------------------------|-----------------------------------------|
| <b><sup>1</sup>H NMR</b>       | Proton nuclear magnetic resonance       |
| <b>2D</b>                      | Two-dimensional                         |
| <b>3D</b>                      | Three-dimensional                       |
| <b><math>\alpha</math>-SMA</b> | Alpha-smooth muscle actin               |
| <b>AFM</b>                     | Atomic force microscopy                 |
| <b>ATP</b>                     | Adenosine triphosphate                  |
| <b>BSA</b>                     | Bovine serum albumin                    |
| <b>CO<sub>2</sub></b>          | Carbon dioxide                          |
| <b>CXCL12</b>                  | C-X-C motif chemokine ligand 12         |
| <b>DAMPs</b>                   | Damage-associated molecular patterns    |
| <b>DAPI</b>                    | 4',6-diamidino-2-phenylindole           |
| <b>DI</b>                      | Deionized                               |
| <b>DMEM</b>                    | Dulbecco's Modified Eagle Medium        |
| <b>DMSO-d6</b>                 | Deuterated dimethyl sulfoxide           |
| <b>D<sub>2</sub>O</b>          | Deuterium oxide                         |
| <b>ECM</b>                     | Extracellular matrix                    |
| <b>EDTA</b>                    | Ethylenediaminetetraacetic acid         |
| <b>FACS</b>                    | Fluorescence-activated cell sorting     |
| <b>FAK</b>                     | Focal adhesion kinase                   |
| <b>FBS</b>                     | Fetal bovine serum                      |
| <b>FITC</b>                    | Fluorescein isothiocyanate              |
| <b>FSC-A</b>                   | Forward scatter area                    |
| <b>FSC-H</b>                   | Forward scatter height                  |
| <b>G</b>                       | Shear modulus                           |
| <b>G'</b>                      | Storage modulus (storage shear modulus) |

|                                   |                                                                 |
|-----------------------------------|-----------------------------------------------------------------|
| <b>G''</b>                        | Loss modulus (loss shear modulus)                               |
| <b>GCP</b>                        | Good Clinical Practice                                          |
| <b>HA</b>                         | Hyaluronic acid                                                 |
| <b>H<sub>2</sub>O</b>             | Water                                                           |
| <b>H<sub>2</sub>O<sub>2</sub></b> | Hydrogen peroxide                                               |
| <b>HGF</b>                        | Hepatocyte growth factor                                        |
| <b>HMGB-1</b>                     | High-mobility group box 1 protein                               |
| <b>IL-1<math>\beta</math></b>     | Interleukin-1 beta                                              |
| <b>IL-6</b>                       | Interleukin-6                                                   |
| <b>IL-10</b>                      | Interleukin-10                                                  |
| <b>LAP</b>                        | Lithium phenyl-2,4,6-trimethylbenzoylphosphinate                |
| <b>M-CSF</b>                      | Macrophage colony-stimulating factor                            |
| <b>MeHA</b>                       | Methacrylated hyaluronic acid                                   |
| <b>MIP</b>                        | Maximum intensity projection                                    |
| <b>MMPs</b>                       | Matrix metalloproteinases                                       |
| <b>MW</b>                         | Molecular weight                                                |
| <b>MWCO</b>                       | Molecular weight cut-off                                        |
| <b>NaOH</b>                       | Sodium hydroxide                                                |
| <b>NHDF</b>                       | Neonatal human dermal fibroblasts                               |
| <b>P/S</b>                        | Penicillin-streptomycin                                         |
| <b>PBMCs</b>                      | Peripheral blood mononuclear cells                              |
| <b>PBS</b>                        | Phosphate-buffered saline                                       |
| <b>PBS--</b>                      | Phosphate-buffered saline without calcium and magnesium         |
| <b>PBS+/+</b>                     | Phosphate-buffered saline with calcium and magnesium            |
| <b>PCL</b>                        | Polycaprolactone                                                |
| <b>PDGF</b>                       | Platelet-derived growth factor                                  |
| <b>PDMS</b>                       | Polydimethylsiloxane                                            |
| <b>PEOT/PBT</b>                   | Poly(ethylene oxide terephthalate)/poly(butylene terephthalate) |
| <b>PFA</b>                        | Paraformaldehyde                                                |
| <b>PSGL-1</b>                     | P-selectin glycoprotein ligand-1                                |
| <b>rcf</b>                        | Relative centrifugal force                                      |

|                                |                                                    |
|--------------------------------|----------------------------------------------------|
| <b>RGD</b>                     | Arg-Gly-Asp (arginine-glycine-aspartic acid motif) |
| <b>RNA-seq</b>                 | Ribonucleic acid sequencing                        |
| <b>ROCK</b>                    | Rho-associated protein kinase                      |
| <b>rpm</b>                     | Revolutions per minute                             |
| <b>SDF-1</b>                   | Stromal-derived factor 1                           |
| <b>TAZ</b>                     | Transcriptional coactivator with PDZ-binding motif |
| <b>TEOA</b>                    | Triethanolamine                                    |
| <b>TGF-<math>\beta</math></b>  | Transforming growth factor beta                    |
| <b>TNF-<math>\alpha</math></b> | Tumor necrosis factor alpha                        |
| <b>VEGF</b>                    | Vascular endothelial growth factor                 |
| <b>w/v</b>                     | weight/volume                                      |
| <b>w/w</b>                     | weight/weight                                      |
| <b>YAP</b>                     | Yes-associated protein                             |

