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Developing stromal instructive niches to promote wound healing

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Abstract

Wound healing is a complex biological process involving various cell types across spatial and temporal phases. Dysregulation in any of these phases, such as in the case of diabetic wounds, burn injuries or implantation of medical devices may result in non-healing wounds, chronic wounds, and/or fibrosis. The accelerated formation of granulation tissue has been studied to promote and accelerate wound healing. Fibroblasts are the prevalent cell type of the granulation tissue and have been shown to play an important role in both healing processes and fibrosis, governed by the nature of biochemical stimuli, e.g., from immune cells such as macrophages, and the physicochemical characteristics of the microenvironment and/or implanted biomaterials. Biomaterial surface chemistry is known to influence phenotype and functions of various cell types including fibroblasts. Hence, the overall aim of this study was to discover new polymer chemistries that regulate/modulate fibroblast behaviour and phenotype, thereby governing the outcome of wound healing.

To study the effect of polymer chemistry on human fibroblasts, a high throughput screening of 300 homo-polymers from a meth(acrylate) and acrylamide library was performed. Polymers that supported cell attachment and spreading, while modulating proliferation and differentiation to myofibroblasts, were identified. Polymers such as poly(tetrahydro furfuryl acrylate) (pTHFuA) promoted cell proliferation while suppressing differentiation, while polymers such as poly(ethylene glycol phenyl ether acrylate) (pEGPEA) suppressed proliferation and promoted fibroblast differentiation to myofibroblasts.

Selected 'hit' polymers from the high-throughput screen were synthesised, characterised, and scaled up onto coupon sized surfaces for further phenotypic

and functional studies. Fibroblasts cultured on these surfaces were shown to adopt distinct anti-fibrotic and pro-fibrotic profiles, by augmenting cytokine secretion and gene expression of ECM components. Moreover, polymers were observed to directly influence fibroblast migration and proliferation, as assessed in a 2D - wound healing assay; cells cultured on pTHFuA and poly(divinyl adipate) (pDVAd) were observed to have a higher rate of wound closure compared to cells cultured on standard TCP.

To elucidate the mechanisms of the observed polymer induced modulation in behaviour and phenotype of fibroblasts, the stiffness, and the thickness of adsorbed proteins from serum supplemented culture medium on scaled-up polymers was investigated. Quantitative analysis revealed that fibroblast phenotype was modulated by adsorption of selective proteins as opposed to stiffness of the polymers or thickness of adsorbed protein layer.

Lastly, the clinical applicability of identified anti- and pro-fibrotic chemistries was pursued by fabricating these chemistries into microparticles, for application as *in situ* scaffolds to accelerate wound healing. The particles supported fibroblast attachment and influenced proliferation. Moreover, fibroblast – microparticle systems were shown to augment fibroblast and macrophage cytokine secretion and gene expression of collagens towards anti-fibrotic and pro-fibrotic outcomes, thereby accelerating wound healing.

In summary, findings from this study demonstrate the ability of polymer surfaces to modulate fibroblast phenotype and behaviour. The observations presented and discussed in this study, provide a framework through which bio-instructive therapeutics to direct outcome of wound healing could be developed.

Publications and Conference Proceedings

Manuscript in preparation

Polymer Chemistry Modulates Fibroblast Behaviour Towards an Anti-Fibrotic Phenotype: A High Throughput Investigation

Arsalan Latif, Laurence Burroughs, Andrew Blok, Graziela P. Figuredo, David A. Winkler, Morgan R Alexander, Amir Ghaemmaghmi

3D bio-instructive microparticles to accelerate wound healing

Arsalan Latif, Adam Dundas, Valentina Crucitti, Francesco Pappalardo, Ricky Wildman, Derick Irvine, Morgan R Alexander, Amir Ghaemmaghmi

Manuscripts published

Awuah, Dennis, Meshal Alobaid, **Arsalan Latif**, Fabián Salazar, Richard D. Emes, and Amir M. Ghaemmaghmi. 2019. “The Cross-Talk between MiR-511-3p and C-Type Lectin Receptors on Dendritic Cells Affects Dendritic Cell Function.” *The Journal of Immunology* 203(1):148–57.

Dundas, Adam A., Valentina Cuzzucoli Crucitti, Simon Haas, Jean Frédéric Dubern, **Arsalan Latif**, Manuel Romero, Olutoba Sanni, Amir M. Ghaemmaghmi, Paul Williams, Morgan R. Alexander, Ricky Wildman, and Derek J. Irvine. 2020. “Achieving Microparticles with Cell-Instructive Surface Chemistry by Using Tunable Co-Polymer Surfactants.” *Advanced Functional Materials*.

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Abbreviations

AFM	Atomic force microscope
AIBN	Azobisisobutyronitrile
AK	Adenylate kinase
ANGII	Angiotensin II
BE	Binding energy
bFGF	Basic fibroblast growth factor
cDNA	Complementary DNA
COPhBF	Bis[(difluoroboryl) diphenylglyoximate]cobalt (II)
COV	Coefficient of Variation
CTGF	Connective tissue growth factor
DAMPS	Damage-associated molecular patterns
DAPI	4,6-diamidino-2-phenylindole
ECM	Extracellular matrix
Ed-A fibronectin	Extra domain – A fibronectin
FBR	Foreign body response
FBS	Fetal bovine serum
FRP	Free radical polymerisation
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hyaluronan
HGF	Hepatocyte growth factor
HRP	Horse radish peroxidase
HTS	High throughput screening

KE	Kinetic energy
KGF	Keratinocyte growth factor
LC-MS	Liquid chromatography tandem mass spectrometry
MEM	Minimum Essential Medium Eagles
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinases
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMN	Polymorphonuclear leukocytes
ROS	Reactive oxygen species
SAM	Self-assembled monolayer
SNR	Signal to noise ratio
TCP	Tissue culture plastic
T _g	Glass transition temperature
TGF – β 1	Transforming growth factor – β 1
TIMPS	Tissue inhibitors of metalloproteinases
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
XPS	X-ray photon spectroscopy
α – SMA	α – smooth muscle actin

Chapter 1: General Introduction

Chapter 1 - General Introduction

1.1 Tissue Microenvironment: the Extracellular Matrix

Cells in multicellular organisms reside in complex microenvironments with diverse biophysical and biochemical properties. These microenvironments consist of cellular and acellular elements. It is homeostasis between both elements that define normal tissue function. The extracellular matrix (ECM) forms the physical framework providing structural and biochemical support to the cells [1]. The various constituents of the ECM are summarized in table 1.1. In general, the ECM is composed of collagens, fibronectin, vitronectin, elastin, glycosaminoglycans (GAGs) and proteoglycans [2].

Table 1.1: The various constituents of the ECM. Adapted from [2]. The ECM consists of fibre forming and non-fibre forming components.

Components					
Fibre forming collagens	Collagen I	Collagen III	Collagen V		
Other fibre forming proteins	Fibrin	Fibronectin	Vitronectin	Elastin	
Non-fibre forming collagens	Collagen IV	Collagen VI	Collagen VII	Collagen XIV	
Proteoglycans/glycosaminoglycans	Hyaluronic acid	Chondroitin sulfate	Dermatan sulfate	Decorin	Dermatopontin
Matricellular proteins	Osteopontin	SPARC	CCN2	Tenascin – C	Fibulin-5

The ECM guides cellular function by modulating cell morphology, physiological function (by binding growth factors) and by interacting with cell surface receptors to regulate gene transcription and consequently cell phenotype. Although fundamentally the ECM is composed of the elements stated earlier, each tissue has an ECM with unique composition and topology. The differences in ECM traits establish the biochemical and mechanical characteristics of each

organ, such as their tensile and compressive strength, and elasticity. In this thesis, we will focus on the ECM of the skin and their relevance to wound healing.

The ECM components of the skin can be grouped into fibre-forming and non-fibre forming structural components, and matrix cellular proteins [3]. The fibre forming components form a complex three-dimensional structure, while the non-fibre forming components give this structure a charged, dynamic and osmotically active attribute.

1.1.1 Fibre forming components: collagen, fibronectin, vitronectin

The fibre forming component of skin ECM microenvironment predominantly consists of collagens. Other fibre forming components include fibrin, fibronectin, vitronectin, elastin and fibrillin [1], [2].

Collagens provide tensile strength and compressibility to the ECM. Around 28 different collagens have been identified in skin ECM. The majority of which is collagen I and III, however, IV, V, XVII are also present in significant concentrations. Based on these, the collagens can be further divided into fibrillar and non-fibrillar collagen. Fibrillar collagens include collagen I, II, III and V. While non-fibrillar collagens include collagen IV, VI, VII, VIII and XIV. Non fibrillar collagens adhere cells to the basement membranes while assisting other collagens to form fibrils.

Other fibrillar ECM components are fibrin, fibronectin, vitronectin and elastin. Fibrin is a fibrillar protein derived from fibrinogen present in blood plasma. Fibrin forms a provisional clot matrix during haemostatic tissue repair.

Fibroblasts utilize this provisional matrix as a scaffold for cell migration and tissue remodelling. Additionally, studies have implied that fibroblast remodelling (in the proliferative phase of healing) is influenced by both regional mechanical stress and the vector geometry of the initial fibrin matrix [4].

Fibronectin is a dimeric glycoprotein found in soluble form in blood plasma and in an insoluble form in the ECM. After tissue injury and deposition of a fibrin clot, soluble fibronectin assembles into insoluble fibronectin. This involves conformational changes that reveal the matricryptic site RGD (Arg-Gly-Asp) [5]. The RGD binding site stimulates migration of fibroblasts into the wound. Fibronectin has various binding domains that allow its fibrils to interact with cells, other fibronectin fibrils and multiple other proteins within the ECM. Specifically, in wound healing, fibronectin is involved in ECM organization and stability. Studies have shown that fibronectin is crucial for Collagen I deposition and deposition of other structural proteins [4]. Moreover, maintenance of fibrillar organization of collagen requires active polymerization of fibronectin, furthering its importance [4]. The pro-fibrotic cytokine, transforming growth factor- β 1 (TGF- β 1), is known to stimulate fibroblast proliferation. This stimulation is dependent on preassembly of fibronectin [1], [2].

Vitronectin has been shown to be vital in myofibroblast linked contraction of wounds [6]. Fibroblasts adherent to vitronectin were shown to produce fibronectin fibrils that had reduction in exposed RGD sequence. This subsequently led to significantly lower fibroblast proliferation. Elastin is

attributed to elasticity of the ECM in tissues. Though it is known for its structural role, elastin has been profiled to indirectly modulate fibroblast proliferation and collagen I secretion [7].

1.1.2 Non-fibre forming components: proteoglycans and GAGs

Proteoglycans consists of a core protein that is linked to a glycosaminoglycan (GAG) side chain. GAGs are linear polysaccharides found in the ECM either bound to a proteoglycan chain or independently. This section will discuss the role of hyaluronan, decorin and dermatopontin in the ECM, specifically in wound healing.

Hyaluronan (HA) is the most common GAG in the ECM and is involved in maintaining tissue hydration and osmotic balance. HA is known to have key roles in both fibrotic and wound healing processes. The size of HA molecules effects their function in relation to fibrotic and wound healing process [8]. The native high molecular weight HA (>500kDA) is associated with decreased inflammation, increased expression of collagen III and increased activity of anti-fibrotic TGF- β 3. While fragmented HA (<400kDA) is linked with increased inflammation, collagen I expression and increased proliferation of fibroblasts [8], [9]. Furthermore, it traps pro-fibrotic TGF- β 1 molecules in proximity to the fibroblasts, thereby creating a positive autocrine loop differentiating fibroblasts to myofibroblasts. Additionally, specific small sized HA fragments (1kDA) stimulate fibroblast migration and promote wound closure, while decreasing myofibroblast differentiation and fibrosis [10]. Based on these findings the small sized HA fragments are of interest for wound healing.

Decorin is a proteoglycan bound to variable number of GAG chains. Decorin is the most abundant proteoglycan in skin ECM. It has an affinity to bind to TGF- β 1 (which increases when chondroitin and dermatan side chains are removed) thereby acting as natural TGF- β 1 inhibitor [11]. In mice knockout models, the absence of decorin results in increased fibroblast adhesion to collagen or fibronectin, proliferation and migration [12], [13]. These results highlight the role of decorin in decreased fibrosis of healing wounds. Dermatoapontin is another proteoglycan that increases skin elasticity, tensile strength and collagen fibrillogenesis [14]. Furthermore, it has been shown to increase fibroblast adhesion to fibrin matrix and promote fibronectin fibril formation. It has been suggested that decorin and dermatoapontin counter each other to balance fibroblast migration in healing wounds.

1.2 Fibroblasts and Myofibroblasts

Fibroblasts are mesenchymal cells derived from the embryonic mesoderm tissue. They are found widely spaced in the ECM of the connective tissue which serves to connect and support all other tissues including the parenchymal tissue of organs. The most accepted definition of fibroblasts is based on *in situ* morphology. Fibroblasts are recognized to have a spindle-shaped body with cellular processes extending from each tip [15], (Figure 1.1).

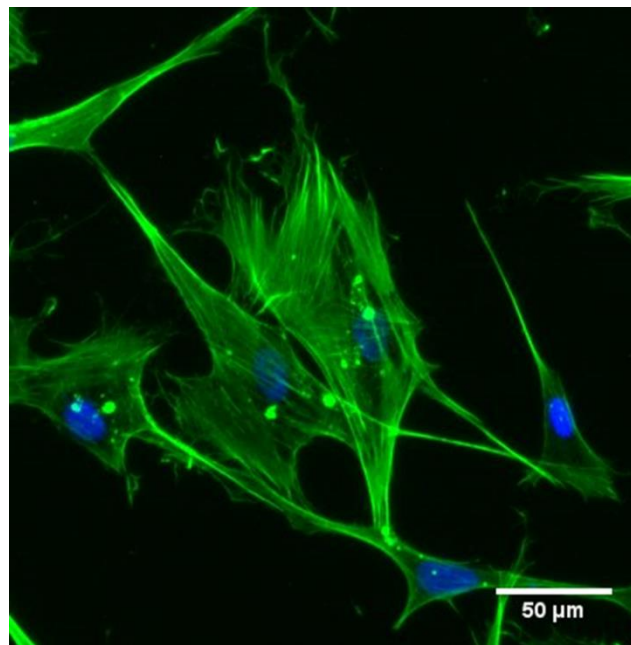


Figure 1.1: Fluorescence microscopy image of fibroblasts. The cells have characteristic spindle like morphology with cellular processes at its ends. Blue: nuclei; green: F-actin

Further, the cells are easily isolated in culture from tissues via several passages on plastic. Applying this definition yields a high degree of heterogeneity in expression and phenotype between tissues, and within the same tissue. Though there are numerous non-specific fibroblast markers, as shown in table 1.2, many are transiently expressed or exclusively expressed in fibrotic context [16]–[18]. To date a universal fibroblast specific marker has yet to be identified.

Table 1.2: List of nonspecific fibroblast markers. Adapted from [15].

Nonspecific fibroblast markers	
Collagen I	Intermediate filament (IF) proteins
Discoidin domain receptor 2 (DDR2)	Platelet-derived Growth Factor (PDGF)
Fibroblast Growth Factor (FGF)	Periostin
Transcription Factor 21 (Tcf21)	Thy-1 (CD90)

Irrespective of the tissue source of fibroblasts, the cells have 4 defined roles: **i)** homeostasis of the ECM by secreting structural proteins and reabsorption by secreting matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) **ii)** inflammation **iii)** angiogenesis **iv)** wound healing and fibrosis [19]. In these roles, fibroblasts interact with their microenvironment and communicate with other cell types via paracrine and autocrine signalling. These are detailed in subsequent sections.

Fibroblasts are not terminally differentiated cells. In a healing wound, responding to microenvironmental cues, such as stiffness and growth factor TGF- β 1, they differentiate into myofibroblasts. The myofibroblast phenotype is characterized by **i)** neo expression of α -smooth muscle actin (α -SMA) **ii)** upregulated ECM expression; collagens and extra domain – A (Ed-A) fibronectin **iii)** contractile nature and **iv)** resistance to apoptosis [19]–[21]. Myofibroblasts originate from multiple sources, including but not limited to fibrocytes, epithelial cells, endothelial cells and multipotent monocytes [15], [17], [21], [22] these are highlighted in figure 1.2. In healing wound, myofibroblasts are especially important in contracting the wound site and laying down ECM scaffold for healing. Additionally, they have increased expression of cytokines, chemokines,

and surface receptors. Both fibroblasts and myofibroblasts have a vital role to play wound healing and fibrosis.

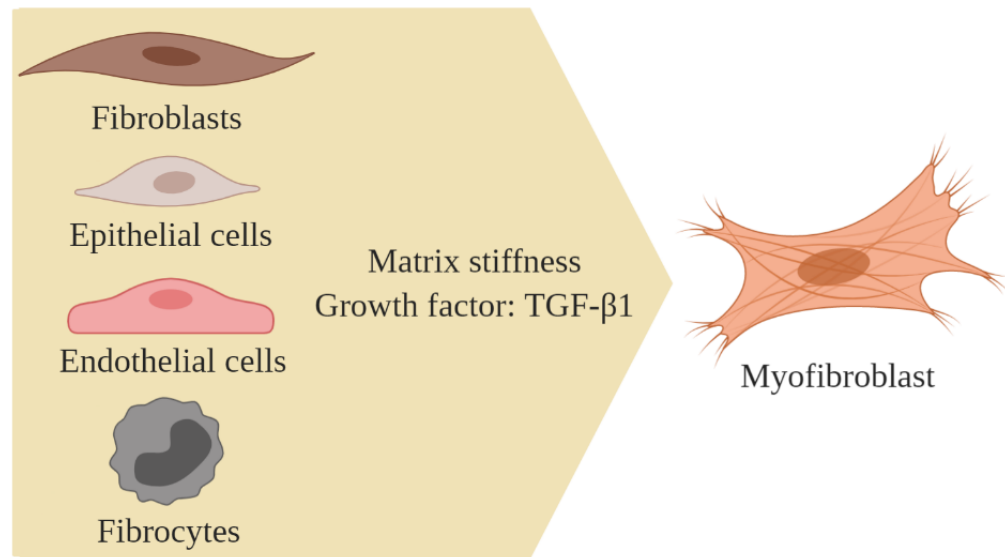


*Figure 1.2: **Cellular source of myofibroblasts.** In response to matrix stiffness and growth factors such as TGF- β 1, fibroblasts and other cell types differentiate into myofibroblasts. Myofibroblasts are α -SMA positive cells that secrete higher amounts of collagens.*

1.3 Wound healing and fibrosis

Wound healing is a complex process involving various cell types across a spatial and temporal matrix. It is achieved through 4 highly precise, programmed and overlapping phases: **i)** haemostasis **ii)** inflammatory phase **iii)** proliferative phase and **iv)** tissue remodelling or fibrosis, (Figure 1.3) [20], [23].

Briefly, in response to tissue injury clotting occurs forming a provisional fibrin matrix during the haemostasis phase. After which, in the inflammatory phase, platelets and immune cells secrete cytokines, chemokines and growth factors promoting chemotaxis of stromal cells. In the proliferative phase of wound healing, the granulation tissue is formed. The granulation tissue consists of immune cells, endothelial cells, fibroblasts and myofibroblasts. The fourth phase of healing, tissue remodelling, involves progressive remodelling of the

granulation tissue. The major component of granulation tissue, collagen III, is progressively replaced by collagen I. Proteolytic enzymes, MMPs and TIMPs, play a major role in this transformation. These phases and their biophysiological functions must occur in the proper sequence, at a specific time and continue for a specific duration at an optimal intensity for normal wound healing [24]. The four phases of wound healing are further discussed in subsequent sections.

The deposition of ECM components, such as collagen and fibronectin, is an integral and reversible part of wound healing. However, normal tissue repair can progress into irreversible fibrotic response if the tissue injury is severe, repetitive, or if the wound healing response is dysregulated. Fibrosis is a hallmark of numerous pathological conditions and is subsequent to dysregulated healing processes after tissue injury, inflammatory conditions and the foreign body response [19], [25], [26]. Fibrosis in a given tissue almost always leads to the loss of its function and is linked to up to 45% of deaths in the developed world [1]. Prime examples include cirrhosis and inflammatory lung diseases. Fibrosis is a condition marked by the excessive accumulation of ECM components around an inflamed or damaged tissue. Factors contributing towards progressive fibrosis include genetic disorders, persistent infection or inflammation, poor circulation, obesity and foreign body responses (in response to implantation of medical devices). Regardless of the initial events, the common thread to all fibrotic diseases is enhanced fibroblast remodelling and failure of myofibroblasts (the primary wound repair cell) to undergo timely apoptosis or dedifferentiation to fibroblasts.

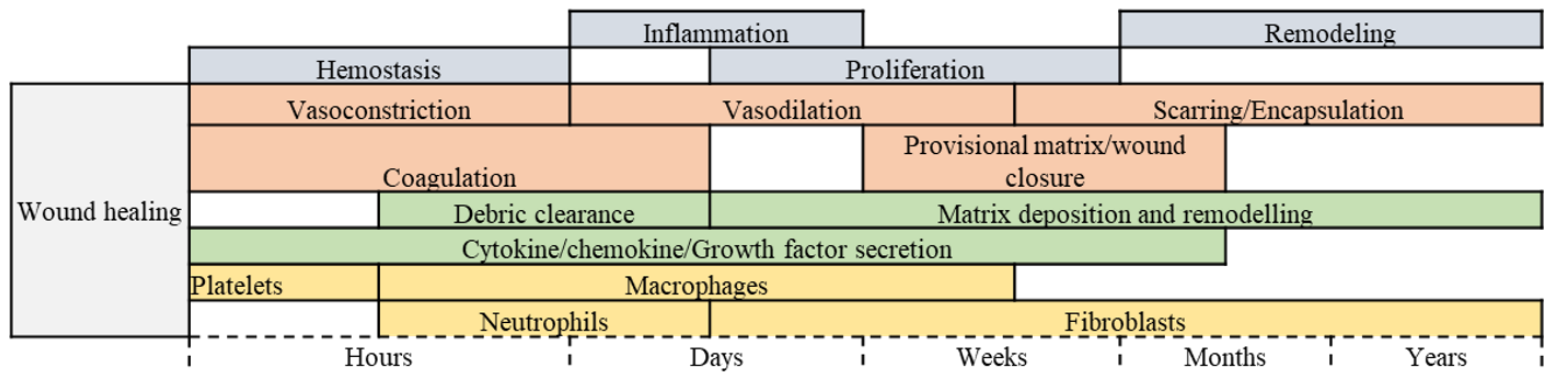


Figure 1.3: **Timeline of wound healing and foreign body response.** Blue, phases of wound healing; Red, gross-scale tissue phenomena; green, cellular activity; gold, major cell types involved.

Adapted from [15].

1.3.1 Haemostasis

Wounding due to injury, surgical and invasive procedures involving biomaterial-based constructs result in vasoconstriction, primary haemostasis, and secondary haemostasis. The vasoconstriction of blood vessels serves to restrict bleeding. This is followed by primary and secondary haemostasis which occur via two concurrent and mechanistically intertwined pathways [27]. Primary haemostasis involves platelet aggregation and the formation of a platelet plug, which is catalysed by exposure of collagen within the wound matrix. Alongside, the secondary haemostasis involves the activation of the coagulation cascade, where soluble fibrinogen is converted into insoluble fibrin strands, which formulate the fibrin mesh. The combined formation of a platelet-plug and the fibrin mesh is called the thrombus. This functions to stop bleeding, release complements, cytokines and growth factors, while providing a provisional scaffold matrix of infiltration of immune and stromal cells [28], [29].

1.3.2 Inflammatory phase: the innate immune response

1.3.2.1 Polymorphonuclear leukocyte activation

Immediately following injury and thrombus formation, polymorphonuclear leukocytes (PMN) such as neutrophils, migrate from the blood towards the wound site. PMN chemotaxis towards the injury site is derived from chemoattractant released from activated platelets and endothelial cells. Further, histamine release from mast cell degranulation has been shown to instigate chemotaxis of PMN and monocyte to implanted biomaterial [30]. The PMNs interact with the damaged-associated molecular patterns (DAMPS), lipid

mediators, and released chemokines by degranulation while triggering a phagocytotic response. This phagocytotic response consists of secretion of proteolytic enzymes and reactive oxygen species (ROS) in order to enhance pathogen removal [31]. These cytotoxic components adversely react by damaging surrounding tissue, thus prolonging the inflammatory response.

PMNs release interleukin-8 (IL-8), CCL2 and CCL4, which they synthesize upon activation [31]. While the former, acts to recruit further PMNs [32], the latter two act as chemoattractants and activation factors for monocytes, macrophages, immature DCs and lymphocytes [33]. An increase in secretion of these chemokines suppresses PMN infiltration in favour of mononuclear cell influx. Due to the lack of further activation signals, PMN undergo apoptosis and are phagocytized by macrophages [34].

1.3.2.2 Macrophages: as inflammatory mediators and wound healing regulators

Macrophages are vital immune cells involved in wound healing and tissue regeneration. These cells control tissue regeneration through phagocytosis of wound debris, secretion of enzymes, cytokines and growth factors; that induce migration and proliferation of fibroblasts [35]. Based on their homeostatic functions, macrophages are classified into classically activated (M1-like) and alternatively activated (M2-like), where the latter is further divided into regulatory and wound healing phenotypes, shown in figure 1.4 [36].

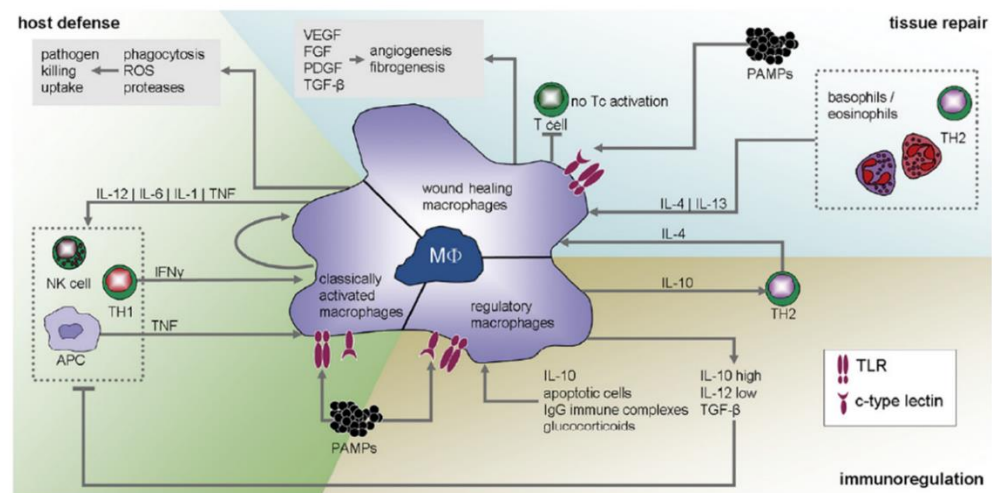


Figure 1.4: **Macrophage polarization.** Adapted from [51].

Macrophages polarise into these different subsets in response to chemical stimuli released by dying cells, innate immune system and stromal cells following injury or infection, and members of the adaptive immune system such as T cells [36]–[38]. With respect to the polarized macrophage subsets; pro-inflammatory M1-like activation of macrophage is a result of secretion of IFN-γ from natural killer cells (innate immune system), TH1 cells (adaptive immune system), and by TNF-

α from APCs (monocytes and DCs) [22]. Pro-inflammatory macrophages exhibit an increase in secretion of inflammatory cytokines, such as IL-6, IL-8, IL-1 β , along with ROS. Moreover, monocytes arriving at the wound site undergo a phenotypic change and differentiate into macrophages. Their activation leads to further release of chemo attractants for inflammatory cells such as IL-8, MCP-1 and MIP-1 β . Macrophages at the wound site exhibit phagocytic ability, secrete inflammatory cytokines, ROS and degradative enzymes; associated with classical activated M1-like macrophage phenotype. The continued presence of pro-inflammatory macrophages causes transition of acute inflammation into chronic inflammation.

Anti-inflammatory M2-like, wound-healing macrophages are polarised in response to IL-4 secreted by basophils, mast cells and granulocytes of the innate immune system or by T_H2 cells of the adaptive immune system. The switch to wound-healing macrophages causes down-regulation of pro-inflammatory mediators and promotes wound healing processes by contributing to production of ECM and by activation of fibroblasts into myofibroblasts [21], [39]. The third subset, regulatory macrophages arise in response to signals from apoptotic cells, immune complexes, glucocorticoids and IL-10 [40]. These macrophages aim to dampen immune responses by secreting high levels of immunosuppressive cytokine: IL-10. Furthermore, adhered macrophages secrete proteolytic enzymes; MMP – 2, -8, -9 and -13 [36]. These enzymes are involved in remodelling of ECM and indirectly effect migration and differentiation of

immune cells. Specifically, increased levels of MMP-9 is speculated to be an indicator of inflammation and poor wound healing [36].

During the early phase of wound healing, macrophages adopt a pro-inflammatory M1-like phenotype which is characterized by release of IL-6, IL-1 β , TNF α , IL-8, MCP-1 and CCL5. With the resolution of inflammation, the M1-like macrophages transition into an M2-like phenotype. This is characterized by the downregulation of inflammatory cytokines, and upregulation of anti-inflammatory, pro-fibrotic mediators such as TGF- β , IL-10, PDGF, and IL-1 α . As mentioned before, an inflammatory response to an injury or infection invokes the action of all three types of macrophages; where pro-inflammatory M1-like macrophages are abundant in the early phase, and regulatory, wound healing anti-inflammatory M2-like macrophage are found in the resolution stage. Aberrations in macrophage function at any of these stages may lead to excessive activation of cells and ECM deposition, resulting in fibrosis[27]. These aberrations range from diminished macrophage numbers during the inflammatory phase to over-stimulation of macrophage-based cell activation.

1.3.2.3 Proliferative phase: granulation tissue formation

The anti-inflammatory M2-like macrophages secrete effector cytokines such as platelet derived growth factor (PDGF) and TGF- β 1 that stimulate fibroblast and endothelial cell migration, differentiation and overall tissue remodelling to form the granulation tissue. This tissue is composed of cellular and acellular components. The acellular component of the early granulation tissue consists of high levels of fibronectin, vitronectin, and collagens type III, IV, V and VI [41].

In the remodelling stage of wound healing, these are replaced by collagen I. The cellular component of granulation tissue consists of activated macrophages, fibroblasts, myofibroblasts and endothelial cells. The latter directing neo-angiogenesis. The acellular components (specifically collagens) of the granulation tissue are secreted by fibroblasts and myofibroblasts and may appear as early as 3 -5 days after injury.

1.3.2.3.1 Fibroblasts and myofibroblasts: towards wound healing or fibrosis

Fibroblasts and myofibroblasts are the prevalent cells of the granulation tissue. In the early stages of the granulation tissue formation their role is to deposit structural ECM components, such as fibronectin and collagen III, to replace the provisional fibrin matrix formed in hemostasis. In the later stages this role shifts to reorganization, where fibroblasts and myofibroblasts secrete MMPs and collagen I. In addition to these, fibroblasts serve an important role beyond that of ECM deposition. This encompasses indirect roles in the inflammatory phase and supporting neovascularization in the granulation tissue. These roles are detailed in the proceeding section.

Fibroblasts respond to microenvironmental stimuli, such as matrix rigidity, soluble factors, biomaterial chemistry and topography by differentiating into myofibroblasts. Chemically, the secretion of TGF- β 1 and PDGF from alternatively activated macrophages and FBGCs promote the activation of resident fibroblasts and fibrocytes, as well as their differentiation into myofibroblasts [53], [54]. These cells secrete high amounts of collagen I, collagen III and fibronectin [55], [56]. It is known that the presence, absence or

ratio of the specific fibrillar collagen types influences fibroblast behaviour (migration, proliferation and fibroblast differentiation to myofibroblasts) [42]–[44]. It was observed that mice with genetic deficiency in collagen III had greater myofibroblast populations. Furthermore, multiple studies have reported that scarless fetal wound healing is associated with a higher ratio of collagen III to collagen I in small and large mammal models.

Myofibroblast activation, proliferation and survival are mediated by a variety of cytokines, such as IL-1, tumour necrosis factor (TNF), TGF- β 1, PDGF and IL-13, and physical factors, such as mechanical stress and stiffness. Myofibroblast differentiation is a two-step process: first, in response to increasing rigidity in the microenvironment, fibroblasts will differentiate into a precursor cell called proto-myofibroblast [58]. Second, in response to TGF- β 1 secreted by the alternatively macrophages, the proto-myofibroblast differentiates into an α -SMA expressing myofibroblast. Additionally, myofibroblasts themselves secrete latent TGF- β 1 [54]. Together with a binding protein, latent TGF- β is bound to ECM components, which with help of myofibroblast contraction and MMPs (MMP-2 and MMP-9), are cleaved to release TGF- β [1], [48], [57].

Continuous activation of myofibroblasts and sustained secretion of ECM components lead to the formation of a fibrous capsule, which is a dense, hypocellular, avascular collagenous network, and is a result of impaired wound healing. The formation of hypertrophic scars as result of healing from burn injuries, trauma, and surgical procedures is a physiological example of excessive myofibroblast activation[27]. Furthermore, fibrosis is the major cause of medical

device failure which is best exemplified in the case of sensors and pacemakers segregated by a fibrotic capsule [18], [57].

1.3.2.3.2 Soluble signals: paracrine and autocrine signalling factors effecting fibroblasts

Apart from producing structural ECM, both fibroblasts and myofibroblasts, are capable of influencing the microenvironment of the healing wound through paracrine and autocrine signals. In turn these cells are influenced by paracrine signals from other cell types in the granulation tissue, (Figure 1.5).

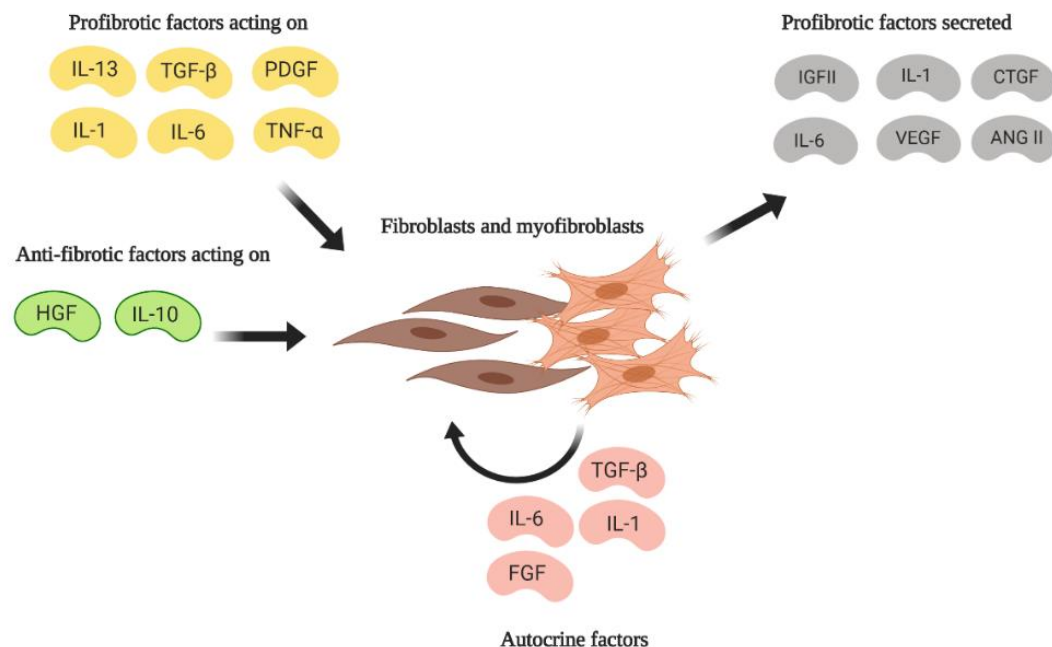


Figure 1.5: **Paracrine and autocrine soluble factors acting on or expressed from fibroblasts.** Adapted from [19].

Fibroblasts participate in the inflammatory phase by secreting CC and CXC chemokines, such as MCP-1 and MIP. These chemokines actively encourage chemotaxis of monocytes and macrophages to the wound site. Moreover, fibroblasts in the granulation tissue have close interactions with the endothelial

cells to facilitate angiogenesis. Endothelial cell migration and proliferation is supported by fibroblast secreted basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF). Furthermore, fibroblast mediated vascular endothelial growth factor (VEGF) directly promotes endothelial cell angiogenesis.

The prototypical pro-fibrotic cytokine is TGF- β . Though there are different isoforms of TGF- β , the two most dominant being TGF- β 1 and TGF- β 3 [22]. TGF- β 1 being the pro-fibrotic isoform. The balance between TGF- β 1 and TGF- β 3 is key for successful wound healing, as TGF- β 1 promotes fibroblast proliferation, differentiation and upregulates collagen I and III expression [45] whilst TGF- β 3 promotes non-fibrotic tissue repair [45]. Both isoforms are secreted by fibroblasts, myofibroblasts, monocytes and macrophages.

A number of other cytokines that co-factor with TGF- β 1 have been identified as pro-fibrotic. The renin-angiotensin-aldosterone system stimulates myofibroblasts and macrophages to produce angiotensin II (ANGII), which upregulates TGF- β 1 expression [46]. Additionally, it stimulates fibroblast proliferation and differentiation to myofibroblasts and stimulates secretion of other pro-fibrotic cytokines such as connective tissue growth factor (CTGF).

CTGF is a matricellular protein involved in angiogenesis, cell migration, adhesion, proliferation, wound repair and ECM regulation [22], [47], [48]. It is highly expressed by endothelial cells in response to a variety of stimuli and is secreted by fibroblasts. The pro-fibrotic cytokine TGF- β 1 induces fibroblasts to secrete CTGF. On its own CTGF is a weak pro-fibrotic cytokine, however as

cofactor with TGF- β 1 it stimulates fibroblasts to upregulate collagen I synthesis and fibroblast differentiation to myofibroblasts[47], [49]. Furthermore, this downstream mediator of fibrosis participates in a positive feedback loop for TGF- β 1 and VEGF production [50].

The Fibroblast Growth Factor (FGF) family consists of several related polypeptides including acidic FGF (aFGF), keratinocyte growth factor (KGF) and bFGF. The latter having an important role in wound healing. Despite bFGF having multiple cellular targets, it requires heparin or heparin sulfate to bind their surface receptors [19], [51]. As a result they are found close to ECM producing fibroblasts. It is secreted by endothelial cells, keratinocytes and fibroblasts, and works in an autocrine and paracrine signalling loop to promote proliferation and migration of these cell types. Interestingly, upregulated levels of bFGF have been associated with accelerated healing via accelerated granulation tissue formation [52].

PDGF is expressed by platelets, endothelial cells and macrophages. It plays a role in inflammation, angiogenesis, wound healing and fibrosis; where it induces fibroblasts to proliferate and differentiate into myofibroblasts[46], [49]. The latter contracting the ECM. IL-6 is classified as a pro-inflammatory cytokine secreted by T-cells, macrophages and fibroblasts. It targets multiple cell types including macrophages and fibroblasts; augmenting collagen synthesis and is able to downregulate α -SMA expression [19], [53]. Furthermore, it has roles in immune cell infiltration and angiogenesis. Anti-fibrotic soluble factors include

hepatocyte growth factor (HGF) which has important roles in wound healing and angiogenesis [19].

1.3.2.4 Tissue remodelling

The final step of successful wound healing is remodelling and regeneration of wound site, such that it resembles native tissue. In the absence of continuous stimuli, the granulation tissue remodels to form scar tissue. The formation of scar tissue is known as the physiological end point of mammalian wound healing [27], [54].

The shift from granulation tissue to scar formation diminishes the cellular presence at the wound site, thus making the wound site hypocellular. Fibroblasts differentiate into myofibroblasts, which contract the wound site decreasing the surface area of the scar tissue. Additionally, these cell types secrete MMPs and TIMPs, that degrade the ECM thus remodelling it. As the MMPs degrade the ECM, the secretion of new ECM components is slowed [27]. Thus, the basket-weave like collagen III, composing the granulation tissue, decreases and is replaced by organized, isotropic and higher tensile strength collagen I.

Following ECM remodelling, the TIMPs block MMPs, thus halting further ECM degradation. The myofibroblasts at the wound site undergo apoptosis or dedifferentiate to fibroblasts [27], [55]. The continued presence of myofibroblasts in the remodelled tissue leads to formation of hypertrophic scars or into fibrotic tissue. This abnormal behaviour has been associated with increased mechanical loading during the healing phases, thus providing a mechanotransductive feedback promoting the myofibroblast phenotype, and

subsequent excessive ECM expression[20], [27], [56]. Moreover, myofibroblasts in fibrotic tissue have been shown to overexpress the “do not eat me” CD47 marker. Thus, instructing macrophages not to phagocytize myofibroblasts resulting in increased numbers of myofibroblasts and excessive ECM secretion [57].

1.3.3 Biomaterial mediated fibrosis: the foreign body response

Biomaterial-mediated fibrosis is similar to physiological fibrosis in that it leads to a highly fibrous and hypocellular matrix composed of collagen I and III [15], [44]. The host reaction to biomaterial implantation is called the foreign body response (FBR). The FBR determines the success of integration and biological performance of implanted medical devices. Following implantation of a biomaterial, the body attempts to heal the wound by sequentially causing acute

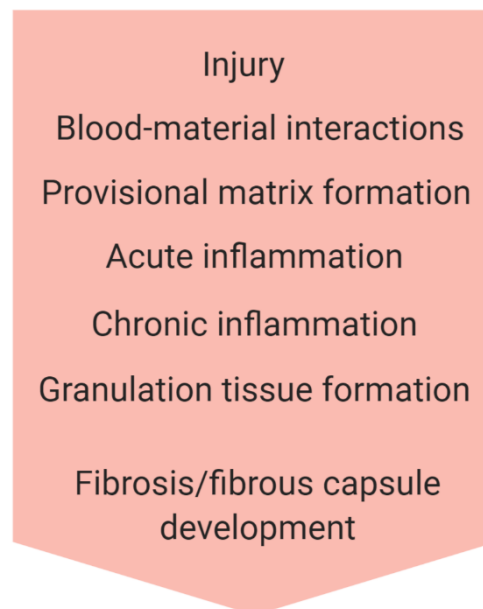


Figure 1.6: Sequence/continuum of host reactions following implantation of medical devices. Adapted from [12]

inflammation, granulation tissue formation and the inevitable formation of fibrous tissue, summarized in figure 1.6. The FBR is influenced by biomaterial surface properties, mechanical signals, and changes in the microenvironment. Like pathological fibrosis, the cell types involved in FBR encompass a wide variety of cells: neutrophils, macrophages, foreign body giant cells, endothelial cells, and stromal cells.

The wound healing response is dependent on the degree of injury or defect created by the implantation procedure. Based on this, wound healing is divided into two **a)** wound healing by primary union and **b)** wound healing by secondary union [58]. Wound healing by primary union is the healing of clean, surgical incisions in which wound edges have been sutured. This does not constitute wound healing in response to implantation. Wound healing by secondary union occurs when there is a large tissue defect and an extensive loss of cells and tissue. Due to this, a larger granulation tissue is formed that may lead to larger areas of scar formation or fibrosis. Wound healing by secondary union is associated with biomaterial implantation [58]. It is understood that the presence of the biomaterial dysregulates normal wound healing. This dysregulated wound healing leads to formation of a fibrotic capsule around implanted medical devices limiting the devices functionality. Such as in the case of implanted sensors and drug delivery systems, and cochlear implants [22], [25].

The end stage of biomaterial-mediated wound healing almost always leads to formation of a fibrotic capsule around the biomaterial, the latter serves as the foreign body. This isolates the foreign body from the local microenvironment of

the host body. In case of fibrous capsules around breast implants, the predominant cell type are myofibroblasts (27%) followed by FBGCs, polymorphonuclear leukocytes, lymphocytes, plasma cells and mast cells [22]. The acellular component of the fibrotic capsule initially consists of myofibroblast secreted collagen III which is gradually replaced by collagen type I [25], [41]. Collagen III has an important role regulating collagen fibrillogenesis and fibril size. Its importance in early granulation tissue is further cemented by studies that have linked excessive scar formation with decreased collagen III expression. Interestingly, downregulated collagen III expression is observed to increase myofibroblasts. During fibrous capsule formation, like in normal wound healing, the secreted collagen III is gradually replaced by collagen I, which forms the main collagen type of the fibrous capsule. Furthermore, studies have shown that the higher ratio of collagen III to collagen I is indicative of prolonged inflammation and thicker capsule formation [44].

It is understood that the presence of the biomaterial dysregulates normal wound healing. Both fibroblasts and myofibroblasts are effector cells involved in the secretion of ECM, including collagens, from the development of the granulation tissue to the formation of the fibrous capsule. In the case of biomaterial-mediated fibrosis their phenotype and subsequent behaviour are directly modulated by cell-biomaterial interactions and indirectly modulated by immune cells response to the implanted biomaterial, (Figure 1.7). It has been suggested that by modulating fibroblast behaviour through biomaterial surface characteristics, the thickness and formation of the fibrous capsules may be minimized. More so,

fibroblast-instructive dressings and scaffolds may be developed to accelerate healing of chronic wounds.

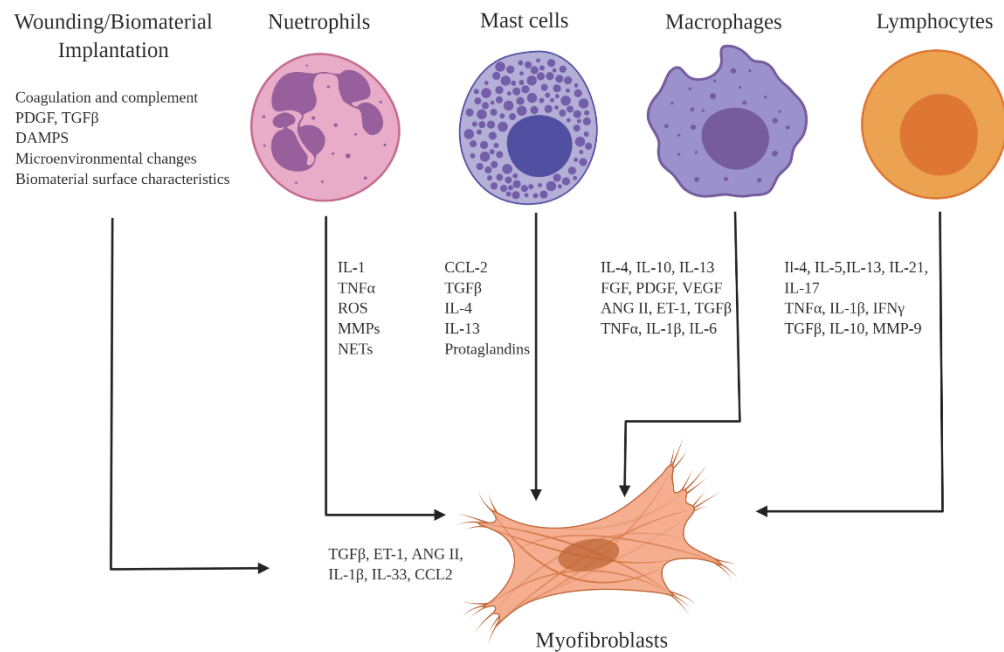


Figure 1.7: *Immune cells secrete cytokines and growth factors that influence myofibroblasts.* Adapted from [22].

1.4 Engineering biomaterials to modulate wound healing and the foreign body response

The outcome of biomaterial implantation is dependent on the extent of the FBR, and the cellular processes involved in inflammation and wound healing. After implantation of a biomaterial, injury to the blood vessels causes extravasation of blood in the vicinity of the biomaterial, initiating a blood-material interaction. The ensuing cascade involves adsorption of proteins, lipids sugars and ions on the biomaterial surface [28], [29], [59]. Through protein adsorption the foreign interface of the biomaterial is translated into a biological language, that the cells interpret. Thus, protein adsorption guides cell attachment, morphology, proliferation and downstream differentiation and protein expression. It can be

inferred that by modulating protein adsorption on biomaterial surfaces, the phenotype and function of cells could be modulated, thus modulating wound healing.

Protein adsorption can be modulated by factors such as surface topography and chemistry [60], [61]. The latter would be further discussed in the following section. Current strategies are directed towards controlling the immune response, specifically modulating macrophage polarization to a pro-inflammatory or anti-inflammatory phenotype [22], [36], [59], [62]. This indirectly influences fibroblast behaviour and subsequent formation of the fibrous capsule[23], [42]–[44]. Though macrophages play an important role in fibrosis, myofibroblasts have been marked as the major players [22]. It has been speculated that by modulating fibroblast behaviour through biomaterial surface characteristics, the wound healing phases could be accelerated by promoting formation of granulation tissue and minimizing the FBR[63], [64].

To this end, there are limited number of studies that have investigated modulating stromal cell behaviour, specifically fibroblast behaviour, in an attempt to guide the foreign body response towards successful, accelerated healing. These strategies encompass the use of a) biomaterial surface characteristics, such as surface topography and chemistry in the form of coatings or scaffolds [27], [60], [61], [77]–[81] and b) controlled release of inhibitors for pro-fibrotic cytokines (TGF- β 1, PDGF, ANG II) [22], [70], growth factors promoting anti-fibrotic, pro-healing phenotypes (FGF2, HGF, EGF and PDGF) [64], [71] or pro-apoptotic drugs targeting myofibroblasts [22], [72], (Figure 1.8).

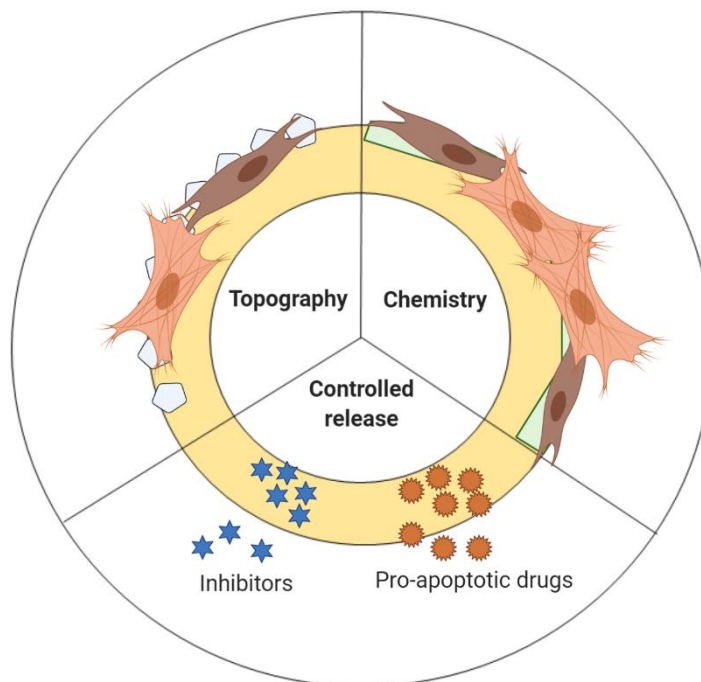


Figure 1.8: Methods to modulate fibroblast and myofibroblast behaviour using biomaterial properties.

Though these methods have been successful *in vitro* their success *in vivo* is varying. Numerous studies have documented controlling adherent cell behaviour through surface chemistry of biomaterials [22], [28], [78], [42], [44], [66], [73]–[77]. Thus, this strategy may be crucial in invoking a regenerative response from fibroblasts and myofibroblasts and could prove to be versatile in application (surface chemistry modifications may be easily applied to a range of medical devices and translated into delivery vehicles, such as microparticles).

1.4.1 Modulating cell behaviour with surface chemistry

Material surfaces can be modified by a variety of methods, such as the application of surface chemical gradients, self-assembled films, surface-active bulk additive and surface chemical reactions [73], [79]. The most common surface modifications are based on using self-assembled monolayers (SAMs)

[73], [80]. These monolayers provide a versatile platform to study the effect of functional groups on cell behaviour. By changing the terminal group on the SAM any functional group can be investigated. Changes in the functional group subsequently change surface properties of the SAM such as wettability and surface charge/polarity. In turn, these properties influence protein adsorption onto the biomaterial surface. The determinants of protein-mediated cell responses are a) the adsorption profile: which proteins are adsorbed and in what quantities and b) protein bioactivity functionality, as determined by conformation, orientation etc [73], [81]. Table 1.3 summarizes the effect of different functional groups on cell behaviour. Studies have shown that NH_3 and COOH group rich surfaces have higher fibroblast attachment than CH_3 group rich surfaces. Additionally, it has been documented that NH_3 and COOH group rich surfaces preferentially adsorb fibronectin and vitronectin from serum [67]. Though CH_3 rich group surfaces adsorb large quantities of proteins, the conformation of these proteins changes due to hydrophobic interactions [42], [67]. Interestingly, increased fibroblast proliferation was noted on surfaces with high concentrations of amine groups: linking the density of amines with proliferation [42], [82].

Table 1.3: Cell behaviour on functional groups of surface chemistries studied. Adapted from [85].

Functional group	Cell studied	Type of study
CH3	Human fibroblasts	Adhesion, spreading, proliferation and differentiation
	Endothelial cells	Adhesion
	Mesenchymal stem cells	Adhesion, proliferation, mRNA expression
	Myoblasts	proliferation and differentiation
	Neutrophils	Adhesion
OH	Human fibroblasts	Adhesion
	Endothelial cells	Adhesion
	Mesenchymal stem cells	Adhesion, proliferation, mRNA expression
	Osteoblasts	Adhesion, mineralisation
NH2	Human fibroblasts	Adhesion, spreading, proliferation, differentiation
	Endothelial cells	Adhesion
	Mesenchymal stem cells	Adhesion, proliferation, mRNA expression
	Myoblasts	proliferation and differentiation
	Osteoblasts	Adhesion, mineralisation
COOH	Human fibroblasts	Adhesion, spreading, proliferation, differentiation
	Mesenchymal stem cells	Adhesion, proliferation, mRNA expression
	Myoblasts	proliferation and differentiation
	Mesenchymal stem cells	Adhesion, proliferation, mRNA expression
RGD	Human fibroblasts	Adhesion, spreading and cytoskeletal organization
	Osteoblasts	Adhesion, spreading and cytoskeletal organization

As previously mentioned, the change in functional groups translates to change in surface wettability and charge. The surface charge on biomaterial surfaces play a significant role in cell-material interactions through adsorbed proteins. Studies have indicated that fibronectin has an affinity to bind to positively charged surfaces [28], [83]. Thus, promoting cell attachment on these surfaces. Additionally, surface charge may directly affect cell behaviour. The negatively charged cell membranes are known to closely adhere to positively charged surfaces. Though cell attachment on positively charged surfaces is higher, subsequent cellular processes such as cell spreading, and differentiation are impaired. This reinforces that integrin engagement with adsorbed proteins is necessary for cell survival, though surface charge may directly influence cell attachment [28], [84].

Studies have noted that hydrophilic surfaces support higher cell attachment than hydrophobic surfaces. This enhanced cell response on hydrophilic surfaces is explained by the difference in proteins adsorbed from serum, blood plasma and/or whole blood. The key components guiding cell attachment are vitronectin and fibronectin. Studies observed that vitronectin competitively adsorbs onto hydrophilic surfaces than hydrophobic surfaces [28], [80], [85]. Though, both vitronectin and fibronectin have been shown to adsorb onto both hydrophilic and hydrophobic surfaces; the cell adhesive functionality of fibronectin has been noted to decrease on hydrophobic surfaces. This suggests an alternate fibronectin conformation on hydrophobic surfaces that does not support cell attachment. Furthermore, it reinforces that the ability of fibronectin to retain functionality on hydrophilic surfaces is an important contributor to normal cell response. Highly hydrophobic surfaces have been known to preferentially adsorb higher amounts of albumin, reducing cell attachment [42], [86]. Moreover, moderately hydrophobic surfaces encourage fibroblasts to secrete higher collagen I to collagen III [42], [44]. Thus, implicating hydrophobic surfaces as contributors to biomaterial mediated fibrosis.

1.5 High throughput approaches to biomaterials discovery

Though SAMs have provided valuable insight into the behaviour of adherent cells on a range of different functional groups, they are limited in terms of the number of materials that can be studied in parallel and are difficult to translate onto medical devices [42]. Conventional methods of identifying novel materials for implantable medical devices encompass repeated cycles of operation, thus limiting and encumbering the discovery process [87].

High throughput screening (HTS) has been used in the pharmaceutical setting with success to facilitate large scale early-stage drug screening. Libraries of compounds can be assayed against biological targets to generate lead candidates for further assessment.

HTS has been translated to the screening of natural and synthetic biomaterials via platforms such as the polymer micro-arrays [87]–[90]. Moreover, the development of the TopoChip has enabled HTS of topographies as well [91]. These platforms are aimed to enable rapid screening of hundreds to thousands of materials in parallel, in order to identify materials that exhibit unique levels of control over cell behaviour. Selected materials can then be scaled up for further biological assessment. The HTS strategy has facilitated discovery of bio-instructive materials in various cases, such as the discovery of polymers that modulate stem cell fate [92], [93], support adhesion and spreading of cardiomyocytes as well as polymers that resist bacterial attachment[89]. In all, the HTS method in comparison to conventional screening methodologies

provides expedited and unbiased analysis process, since it enables assessment of multiple elements on a single platform [92].

1.6 Hypothesis and project overview

It was hypothesized that surface chemistry, specifically polymers, can modulate key functional behaviour of fibroblasts: attachment, proliferation, and differentiation to myofibroblasts. These cell traits have been implicated in governing the outcome of wound healing and fibrosis. Thus, by controlling fibroblast phenotype and behaviour using polymer surfaces the outcome of wound healing and fibrosis could be augmented.

To test this hypothesis, a high throughput screening of a homo-polymer library was performed. The library consisted of approximately 300 homo-polymers from the acrylate, methacrylate, and acrylamide functional groups. The screening assisted in narrowing the best candidates for modulation of fibroblast behaviour. Homo-polymers were assessed against fibroblast attachment, morphology, proliferation, and differentiation into myofibroblasts.

To validate data from HTS experiments, a selection of 'hit' polymers were then scaled up and coated on glass substrates. This involved synthesizing homo-polymers using the thermal free-radical polymerization method and subsequently coating them onto glass substrates. Cell behaviour was validated through cell attachment, proliferation, α -SMA expression. In addition, their secretory profile was studied: cytokines, growth factors and ECM components.

Lastly, the two best performing polymers with a) anti-fibrotic and b) pro-fibrotic properties were selected to be fabricated into microparticles. Microparticles present an opportunity to translate these chemistries into a clinically applicable format for wound healing applications. The modulatory behaviour of fibroblasts and macrophages on these microparticles was then assessed using cytokine profiles and gene expression.

Chapter 2: General materials and methods

Chapter 2 - General materials and methods

2.1 Cellular and molecular techniques

2.1.1 Immunofluorescence assay

Immunofluorescence assay techniques are used to assess qualitative and quantitative distribution and expression levels of biomolecules in biological samples [94]. In short, the technique uses fluorescent labelled antibodies and fluorescence imaging techniques for the detection and visualisation of target biomolecules in cells and/or tissue samples. Specific immunofluorescence techniques used in the project are detailed in subsequent subsections.

2.1.1.1 Immunocytochemistry

The detection/visualisation of target biomolecules in cells or tissue samples is achieved by targeting antigens of interest, such as proteins. The target antigens can be detected directly, direct immunofluorescence, or indirectly, indirect immunofluorescence, (Figure 2.1). Both methods involve use of antigen specific primary antibodies. The direct immunofluorescence method has a fluorophore chemically bound to the primary antibody. Thus, the direct immunofluorescence method is a one-step incubation with the target antigen. On the other hand, indirect immunofluorescence utilizes two antibodies: the unlabelled primary antibody which specifically binds to target antigens, and the fluorophore labelled secondary antibody which binds to the unlabelled primary antibody. The indirect (two step) immunofluorescence method provides flexibility of fluorophore used and yields higher (Signal-to-Noise Ratio) SNR, since multiple secondary

antibodies may bind to a single primary antibody [95]. The antigen target bound fluorophore can then be visualised using a fluorescent microscope.

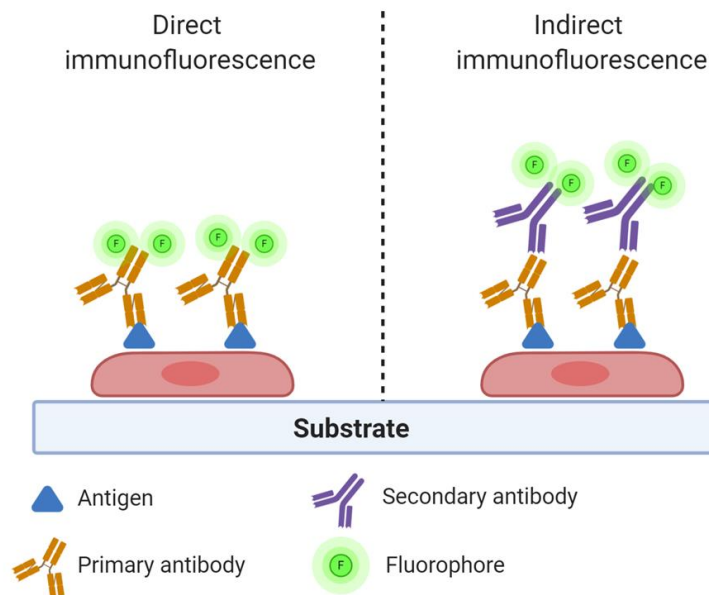


Figure 2.1: **Direct and indirect immunofluorescence methods.** Figure made in Biorender.

2.1.1.2 Fluorescence microscopy

Fluorescence microscopy is a technique used to visualise targeted antigens following immunocytochemistry techniques. The instruments that utilize fluorescence imaging principles include laser scanning confocal microscopes, two-photon excitation microscopes and wide-field microscopes. The selection of the imaging instrument is based on the sample type and imaging requirements. The majority of these microscopes are assembled such that the excitation and emission light pass through the same objective, such microscopes are called epifluorescence microscopes [95].

An epifluorescence microscope consists of a light source, excitation filter, dichroic, objective lens, emission filter and an eyepiece and/or CCD camera. In

brief, the light source generates a wide spectrum of light which passes through excitation filter. The excitation filter transmits the required wavelength of light for that fluorophore while reflecting others. The transmitted light is then reflected towards the sample with the dichroic mirror and is focused on to the sample with the objective. The fluorophores bound to the antibody absorb this excitation light, thereby increasing the energy state of its electrons from the ground state to the excited state. On returning to their ground state, the fluorophore emits light with a longer wavelength than the excitation light: this is called the emitted light. The emitted light is gathered by the objective and is transmitted through the dichroic to the emission filter. The emission filter transmits light of the appropriate

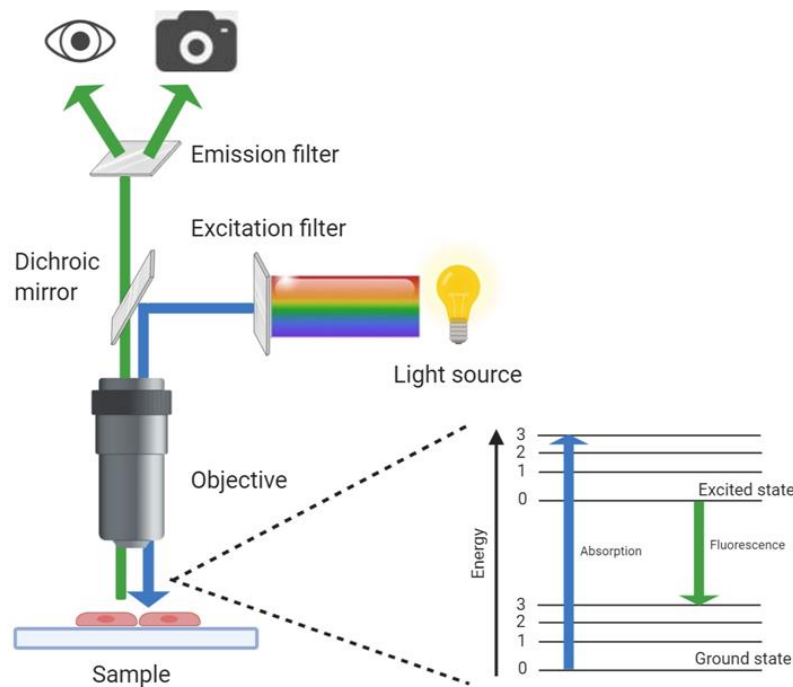


Figure 2.2: **Schematic representation of epifluorescence microscope.** The Jablonkisi diagram shows energy absorption and subsequent release of fluorescence. Figure made in Biorender.

wavelength to the eyepiece or the CCD camera for visualisation of the samples.

The schematic in figure 2.2 summarizes this process.

2.1.2 Enzyme linked immunosorbent assay (ELISA)

ELISA is a highly sensitive and specific immunological technique for detecting and quantifying soluble substances such as peptides, proteins, and antibodies [96]. It involves immobilization of the antigen to a microplate surface which is then complexed with an antibody that is linked with a reporter enzyme. Detection is accomplished by measuring the activity of the reporter enzyme via incubation with appropriate substrate to produce a measurable product. There are several ELISA methods including direct, indirect, sandwich capture and detection methods, (Figure 2.3).

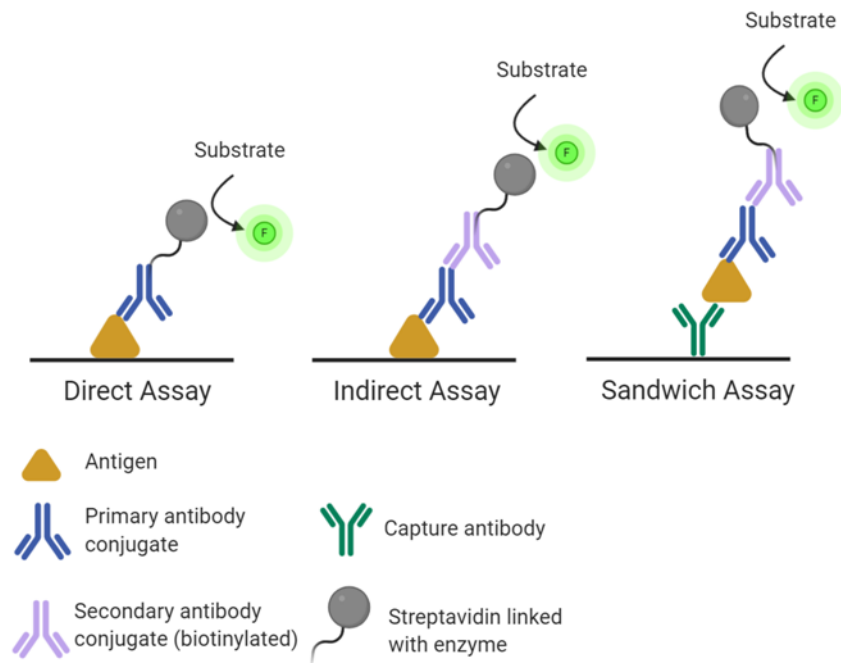


Figure 2.3: **The different ELISA methods include direct, indirect and sandwich assay.** Figure made in Biorender.

The commonly used ELISA method is the sandwich ELISA format which was the approach used in this study. This format involves coating of a capture antibody into a microplate well, on which the antigen or analyte is then captured. A second antibody, the detection antibody having a different specific epitope is then added into the wells. Thus, the analyte is sandwiched between two antibodies, each detecting a different epitope of the antigen. The detection antibody is conjugated with a protein or co-enzyme such as biotin. Following incubation with the antibodies, a detection reagent is added. The detection reagent consists of streptavidin-horse radish peroxidase (HRP), having high affinity for biotin, thus binding to the biotin conjugated detection antibody. The addition of a colourless substrate reagent (tetramethylbenzidine/peroxide) is catalysed into a coloured product with help from HRP. The colour change is proportional to the amount of analyte present. This colorimetric change can then be quantified with a spectrophotometer.

2.1.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical technique capable of amplifying a single DNA molecule into millions of copies within a short time frame [97]. The process is based on the ability of DNA polymerase to synthesize new strands of complementary DNA from single stranded DNA templates. The amplification process involves the following three steps: i) the denaturation step, where the double-stranded DNA is heat denatured to form single-stranded DNA ii) the annealing step, where short DNA fragments called primers align to the single DNA strands iii) extension, here the primers are extended by DNA

polymerase, resulting in two copies of the original DNA strand. These three steps constitute a cycle. A subset method using this principle is the reverse transcription - quantitative PCR (RT-qPCR).

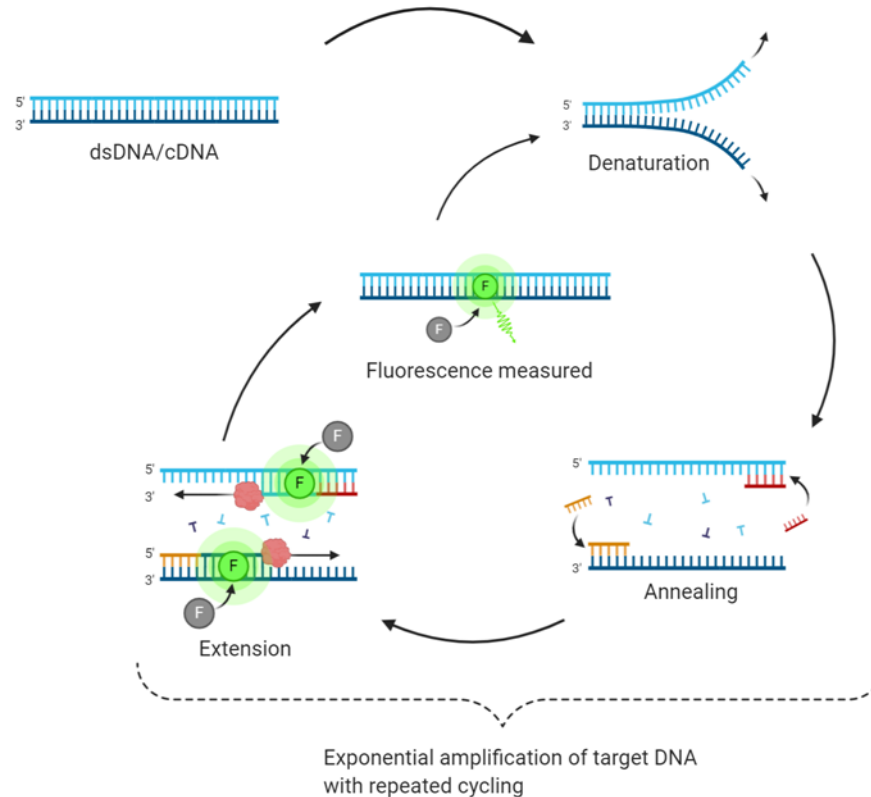


Figure 2.4: Illustration of RT-qPCR steps. RT-qPCR steps include denaturation, annealing and extension. After which fluorescence is measured and the cycle repeated. Figure made in biorender.

The quantitative measure of gene expression in biological samples is mostly performed using the RT-qPCR approach. This approach requires reverse transcription of extracted RNA into complementary DNA (cDNA) using the enzyme reverse transcriptase. The resulting cDNA is subsequently used as template for the qPCR. The qPCR is similar to PCR with addition of fluorescent label, which enables quantification of amplification in real time. The two most used fluorescent systems used in RT-qPCR are the DNA binding, SYBER Green dyes, and the hydrolysis probes, TaqMan probes [98]. This study used the DNA

binding, SYBER Green dyes. These function by emitting fluorescence when intercalated with dsDNA during the extension step of the PCR. The emitted fluorescence is detected at the end of each cycle, where each RT-qPCR run is 30-40 cycles. The detected fluorescence is proportional to the amount target dsDNA present. The method is summarized in figure 2.4.

2.2 Polymer synthesis and casting

2.2.1 Free radical polymerization

Free radical polymerization (FRP) is a method of polymerization by which a polymer forms by successive addition of free-radical building blocks. The reaction mechanism follows three steps: initiation, propagation, and termination. These steps are shown in figure 2.5.

Initiation involves generation of active free radical species through decomposition of thermal or photochemical initiators. In response to heat the thermal initiator is homolytically cleaved producing a pair of free radicals (fragment having unpaired electron). Following its generation, the free radical reacts with the functional group of the monomer (meth(acrylate) or meth(acrylamide) group) to form a chain radical. This is called initiation. The next step of the process is propagation. After the chain radical is generated, it reacts with monomer units turning the whole molecule into another radical. This begins the polymer chain. This radical chain then reacts with other monomer units, progressing its length. The propagation step continues until all monomer units are consumed. However, pairs of radicals have tendency to react with one

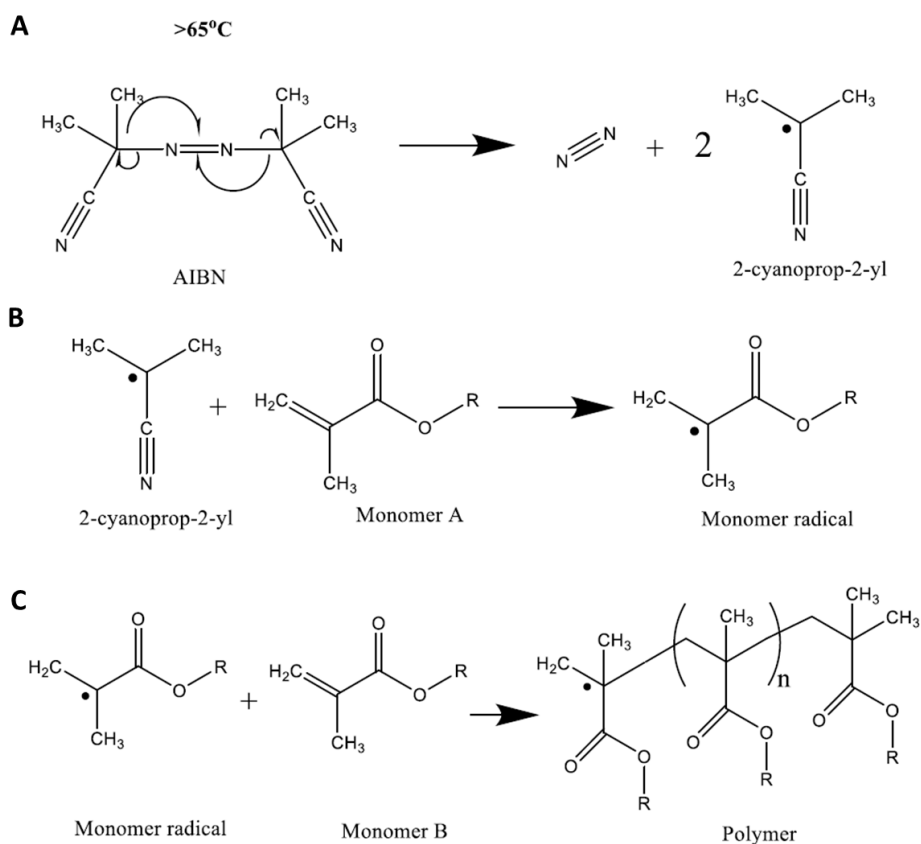


Figure 2.5: **Schematic representation of free radical polymerization steps.** The three steps include A) and B) Initiation followed by C) propagation

another nulling their activity. Termination can occur via combination or disproportionation. In case of the former, two growing polymer chains react with each other forming a single non-reactive polymer chain. Disproportionation occurs when a hydrogen atom is transferred from one radical to the other resulting in two polymers: one with a saturated end and the other with an unsaturated end. In this study, the monomers were polymerized either with photochemical initiators (for polymer microarrays) or thermal initiators (for scaled up studies).

2.2.2 Spin coating

Spin coating is a technique used to fabricate thin films onto substrates. The technique involves application of a solution (in this case a polymer solution) onto a substrate which is then rotated at high speed. The rotation of the substrate at high speed generates a centripetal force that in conjunction with surface tension of the solution pulls the liquid solution into an even coating [99], [100]. The solvent is then evaporated to leave behind a polymer film. These steps are outlined in figure 2.6. The thickness of the film is proportional to the inverse of the spin speed squared [101], as shown in the equation below.

$$t \propto \frac{1}{\sqrt{\omega}}$$

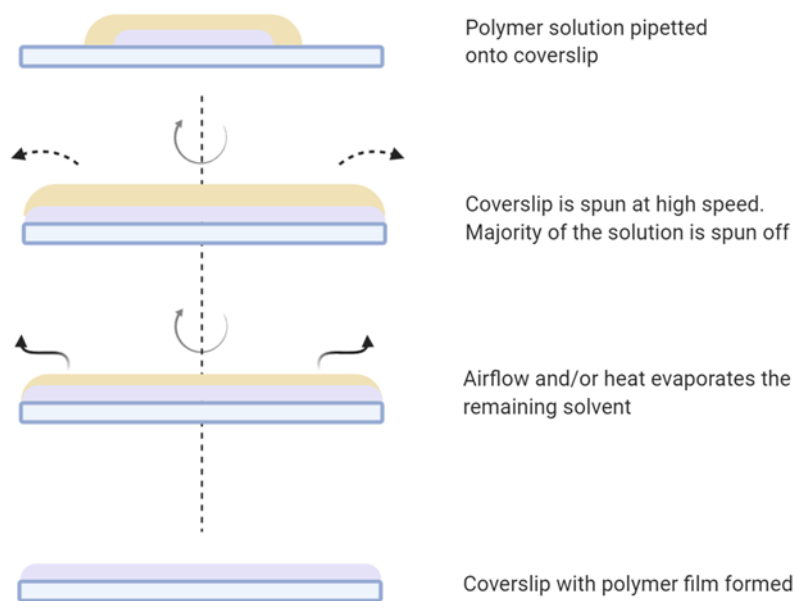


Figure 2.6: **Illustration of the spin coating method.** Polymer solution is pipetted onto a coverslip which is spun at high speeds. The generated centripetal force spreads the polymer solution across the coverslip. Figure made in biorender.

Where, t is the thickness of the film and ω is the angular velocity. For instance, if a film is spun at four times the speed then the film would be half as thick. It should be noted that the exact thickness of the film depends on the polymer concentration and the solvent evaporation rate.

2.3 Surface characterization

2.3.1 Time of Flight Secondary Ion Mass Spectroscopy (ToF-SIMS)

ToF-SIMS is a chemically specific and surface sensitive (ca. 1 nm) analytical method used for surface characterization. The technique uses pulsed ion beams to remove molecules from the top 2nm of the surface for analysis, in a process called sputtering [102]. This causes expulsion of a small proportion of charged atomic or molecular fragments from the outermost surface layers, which are accelerated into the analyser using a voltage and detected measuring their time-of-flight. The ions are detected according to their flight times which is defined by their mass to charge ratio (m/z). Figure 2.7 illustrates these steps.

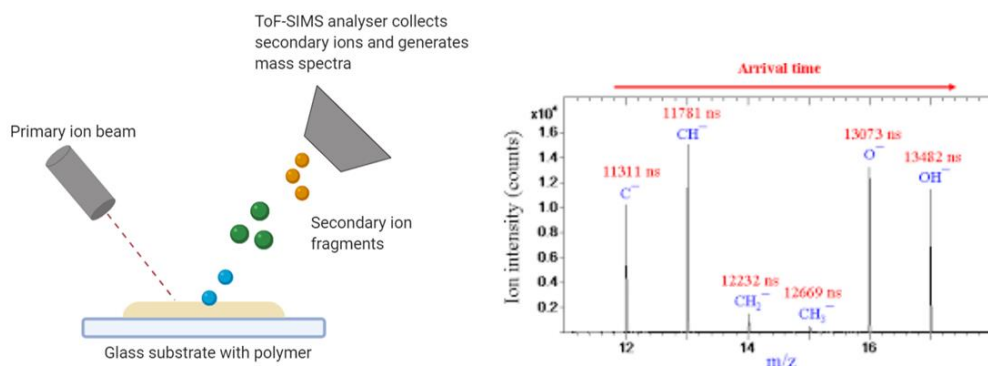


Figure 2.7: **Data acquisition using ToF-SIMS and an example spectrum that is subsequently generated.** Figure made in biorender.

ToF-SIMS spectra are abundant with chemical information as they capture all ions that are created during sputtering. Thus, the technique is very surface sensitive and can identify very similar chemical species. For example, ToF-SIMS has been applied to study protein adsorption studies using the fragmented amino acids[103]. In this capacity it has been employed to generate reference spectra for different proteins, as well as to identify characteristics molecular fragments for each amino acid.

2.3.2 X-ray Photon Spectroscopy (XPS)

XPS is a surface-sensitive (ca. 10 nm) quantitative spectroscopic technique used for measuring surface elemental composition of substrates. This is achieved by irradiating the material with X-rays in an ultra-high vacuum environment. The absorption of X-ray energy by atoms in the material result in ejection of electrons, (Figure 2.8). Where the kinetic energy (KE) of the electron depends upon the photon energy ($h\nu$) and the binding energy (BE).

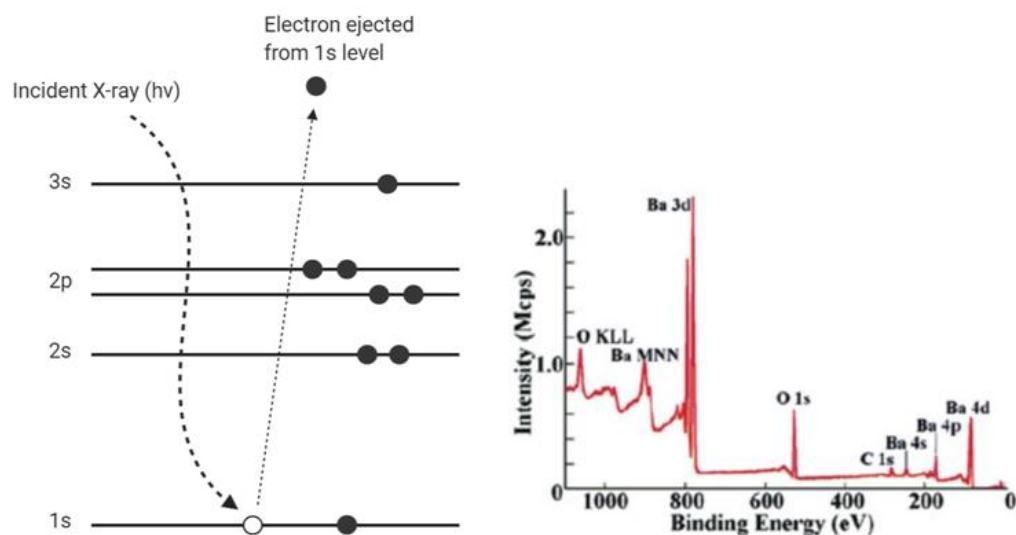


Figure 2.8: **Schematic representation of XPS method.** The irradiation of material surface with an X-ray causes ejection of electrons from the valence shells which are then subsequently translated into an XPS spectrum. Figure made in biorender.

$$KE = h\nu - BE - \omega$$

Here ω is the spectrometer work function. The binding energy is dependent on several factors including the element from which the electron is emitted, the orbital from which it is ejected and the chemical environment of the atom from which the electron was emitted. Thus, the binding energy of an emitted electron can map back to the element from which it originated. The XPS spectrum is a plot of the electron intensity against binding energy; where the intensity of the electrons is proportional to the concentration of elements they originated from. Therefore, the relative amount of elements present on a material can be calculated using the XPS spectrum. For example, XPS has been used to quantitatively measure the protein thickness of adsorbed proteins on biomaterial surfaces [104]. This is accomplished by measuring the overlayer specific elements (nitrogen for proteins) introduced in cell culture on biomaterial surfaces [105].

2.3.3 Water contact angle

The water contact angle is a quantitative measure of the wettability of a solid surface. The technique relies on measuring the angle formed at the intersection

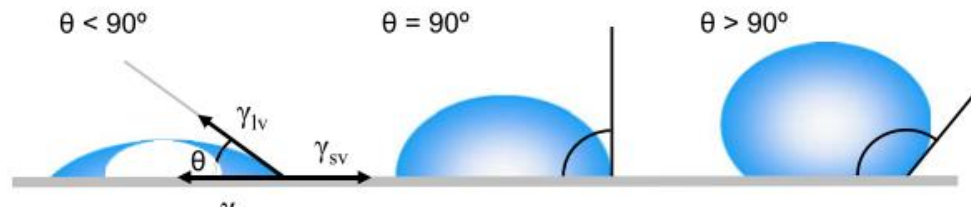


Figure 2.9: Illustration of water contact angle formed on a solid surface with a sessile drop of water. Adapted from [105.]

of the liquid-solid and liquid-vapour interface, which indicates the degree of intermolecular interactions between the liquid and solid phases [106].

The contact angle is determined by placing a sessile drop of water on the surface and measuring the angle between the tangent of the perimeter and the surface at the liquid-solid intersection, (Figure 2.9). The contact angle is related to the solid surface energy and liquid surface tension by Young's equation:

$$\gamma_{lv} \cos \theta_Y = \gamma_{sv} - \gamma_{sl}$$

Where γ_{lv} , γ_{sv} and γ_{sl} represent the liquid-vapour, solid-vapour, and solid-liquid interfacial tensions, respectively, and θ_Y is the water contact angle. A low contact angle is indicative of a surface favouring wettability, while a high contact angle is true for a surface having unfavourable wetting properties.

Chapter 3: Screening for bio-instructive polymers that modulate stromal cell behaviour: a high throughput approach

Chapter 3 – Screening for bio-instructive polymers that modulate stromal cell behaviour: a high throughput approach

3.1 Introduction

The number of implants and medical devices, such as joint implants, neural electrodes, pacemakers, and stents used are steadily increasing as medical science improves and people live for longer [58]. The implantation of such devices often triggers an immune response by the host called the foreign body response (FBR). The FBR initiates chronic inflammation and may lead to fibrotic encapsulation at its end stage; both of which are important concerns in the design of implantable medical devices.

The two dominant effector cells involved in the FBR are macrophages, which participate in the inflammatory stages, and fibroblasts which dominate the proliferative and remodelling stage of wound healing. Both cell types orchestrate the outcome of the FBR: either towards successful wound healing or fibrosis. In this process fibroblasts are directly responsible for producing the acellular capsule around fibrosed materials [15]. Fibroblasts respond to secreted signals from immune cells by migrating towards site of injury, proliferating and upregulating synthesis of extracellular matrix (ECM) components such as fibronectin and collagens [22]. Thereupon, fibroblasts can differentiate into myofibroblasts in the presence of chemical cues such as Transforming Growth Factor – $\beta 1$ (TGF- $\beta 1$) and physical cues such as stiffness. Myofibroblasts are specialized cell types characterised by upregulated expression of ECM

components (collagens) and expression of α -smooth muscle actin (α -SMA). These cell types are necessary for normal wound healing after implantation; however, their persistent presence leads to excessive ECM deposition, which in turn leads to implant encapsulation and fibrosis.

The impact of physical properties of materials, such as surface chemistry and topography, on cell behaviour has been widely studied *in vitro* [42], [43], [74], [82], [92], [107], [108]. Various approaches have been used to modulate cell response away from fibrosis towards successful wound healing and implant integration. These approaches rely on modulation using material surfaces, such as surface chemistry or topography to direct cell shape and thereby phenotype. The abnormal activation of fibroblasts on implant surfaces is a possible major cause for the development of implant encapsulation [109], [110]. Hence, our strategy involved modulating tissue-resident fibroblast behaviour: specifically, attachment, proliferation, and differentiation, using polymer surfaces. In the absence of sufficient knowledge to design such chemical cues a high-throughput approach that allowed examination of a wide chemical space was chosen. High-throughput screening of combinatorial polymer libraries has led to the discovery of polymers that modulated macrophage phenotype to develop immune instructive niches or instructed pluripotent stem cell expansion and differentiation or suppressed formation of bacterial-bio films [92], [93], [111], [112]. These discoveries not only impart the potential of high-throughput screening, but also lead to discovery of bio-instructive properties that otherwise could not be theorised. It was hypothesized that the chemically diverse library of

simple polymers could be screened to identify polymers with stromal cell instructive cues, specifically to modulate fibroblast phenotype and behaviour.

To investigate this hypothesis, a high throughput screening platform, the polymer microarray, was used to screen 300 homo-polymers formed from a (meth)acrylate and (meth)acrylamide monomer library [93]. The polymer microarray enabled the screening of homo-polymers for fibroblast behaviour focusing on attachment, size/spreading, proliferation and myofibroblast marker – (α -SMA) expression. Polymers were selected based on their ability to modulate fibroblast proliferation and differentiation. The approach identified “hit” polymers that could possibly direct host-biomaterial interactions towards a healing and a regenerative outcome.

3.2 Materials and methods

3.2.1 Polymer array fabrication

Polymer microarray were fabricated as previously described [88]. In brief, polymer microarrays were printed onto epoxy coated slides (Xenopore) dip coated with poly (2-hydroxyethyl methacrylate) (pHEMA) (4% w/v, Sigma) in ethanol (95% v/v, in water). Monomers (Sigma, Scientific Polymers and Polysciences) in polymerisation solution were printed using a XYZ3200 dispensing station (Biodot) and metal pins (946MP6B, Arrayit). The polymerisation solutions were composed of monomer (50% v/v) in dimethylformamide with photoinitiator 2,2-dimethoxy-2-phenyl acetophenone (1% w/v). Three replicate spots were printed on each slide. The printing conditions were $O_2 < 2000$ ppm, 25°C, and 35% humidity. To initiate polymerisation, the arrays were irradiated with UV (365nm) for 1 min immediately

after printing and for an additional 10 min at the end of the print cycle. The arrays were sterilized with UV light for 20 minutes prior to cell culture.

3.2.2 Cell Culture

The human lung fibroblasts (CCL-171, MRC-5, ATCC) were cultured in Minimum Essential Medium Eagles (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, non-essential amino acids, penicillin/streptomycin and sodium pyruvate (Sigma). The cells were cultured in T75 flasks at 37°C with 5% supplemental CO₂ until 90% confluent before passaging. For pro-fibrotic culture controls, fibroblasts were cultured with recombinant human TGF- β 1 (10 ng ml⁻¹) (R & D systems) for 96 hours on TCP. All substrates were primed with complete medium for 1 hour prior to cell seeding.

3.2.3 Immunostaining of fibroblasts on polymer arrays

3.2.3.1 Cell adhesion, size, and proliferation

Cells were fixed and permeabilized with 0.15% triton-x (Sigma). To probe for F-actin the cells incubated with Alexa Fluor ® 647 Phalloidin 1:50 dilution (Cell Signalling Technology) for 30 minutes. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cell attachment was measured by counting number of nuclei and cell size quantified by measuring cytoskeletal area. Cell proliferation was quantified by counting number of nuclei at 24 hours and 96 hours of culture.

3.2.3.2 Mean Fluorescence Intensity of α – Smooth Muscle Actin

At 72 hours of culture, the cells were fixed and permeabilized with 0.5% triton-x (Sigma). Non-specific binding proteins were blocked with 10% goat serum for 30

minutes. The cells were stained for anti α -smooth muscle actin 5 μ g/ml (clone 1A4, Sigma) and Rhodamine Red – X ® 5 μ g/ml (Thermofisher Scientific) used as a secondary antibody. Fluorescence intensity was quantified by measuring the integrated density of α - smooth muscle actin positive cells, this was normalized against the total number of cells.

3.2.4 Imaging and image analysis

Mounted samples were imaged using the Zeiss TIRF (Carl Zeiss) device in widefield mode with a 20x air objective. Image analysis protocols to measure cell attachment, size, proliferation, and α -smooth muscle actin were developed using FIJI ImageJ. Details of these protocols are presented in appendix 2.

3.2.5 Data and statistical analysis

All data acquisitioned from the HTS is expressed as mean $\pm$ standard deviation, with $n = 8$ to 12 , $N = 2$. To understand when the mean value was significant relative to the variance observed, a test was applied comparing the mean to measured variance [113]. A signal to noise ratio (SNR) was calculated, which is the ratio of mean value and standard deviation of signal. A SNR of 3 was used as a threshold for differences in cell adhesion, proliferation and α -SMA expression.

3.3 Results

3.3.1 Cell seeding and assay parameters

3.3.1.1 Cell seeding density for polymer microarray

To determine the optimal seeding density on the polymer microarray, a human fibroblast cell line (MRC-5) with a range of cell seeding densities were seeded onto poly-L-lysine coated glass slides. The seeding density ranged from S1, 18×10^3 cells/cm² to S4, 106×10^3 cells/cm². The optimal cell seeding density was assessed by measuring the area covered by adherent cells (cell size) and the number of adherent cells (cell count). The percentage area covered by the cells increased from S1 (18×10^3 cells/cm²) to S3 (101×10^3 cells/cm²), where no

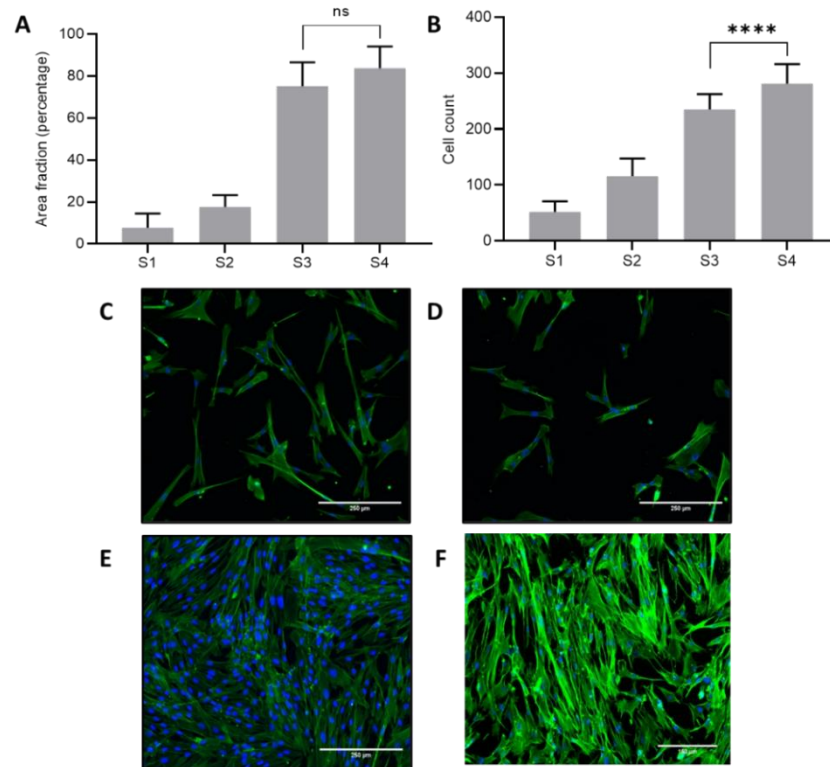


Figure 3.1: **Optimal cell seeding on the polymer microarrays.** This was assessed by A) cell size as measured by area fraction and B) the number of adherent cells. Representative images of cell seeding densities C) S1 D) S2 E) S3 and F) S4. Images representative of 10 images. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

significant difference in coverage was observed between S3 and S4, (Figure 3.1A). A similar pattern was observed for the number of adherent cells, with exception that S4 had significantly higher number of adherent cells than S3, (Figure 3.1B). Based on this data, S3 was chosen as the optimal cell seeding density for the polymer microarray.

3.3.1.2 Impact of TGF- β 1 on α -SMA expression

To study differentiation of fibroblasts into α -SMA expressing myofibroblasts, cells (MRC-5) were seeded into TCP and treated with either 1ng/ml or 10ng/ml of pro-fibrotic cytokine TGF- β 1 for 96 hours. The results show that fibroblasts treated with 10ng/ml of TGF- β 1 had significantly higher expression of α -SMA than the untreated condition, (Figure 3.2A). Though fibroblasts treated with 1ng/ml of TGF- β 1 had higher mean expression than the untreated condition, this

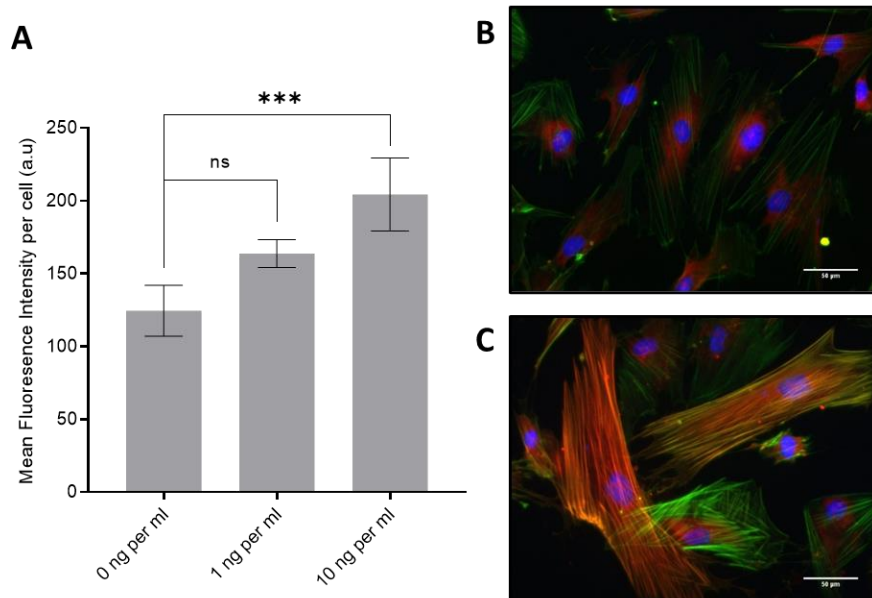


Figure 3.2: Mean fluorescence intensity measurements of α -SMA. A) The 10 ng ml⁻¹ condition had higher intensity than 0 ng ml⁻¹. The representative images of B) 0 ng ml and C) 10 ng ml conditions. Images representative of 10 images. Blue: cell nuclei Green: F-actin and red: α -SMA. All data shown is from two biological replicates with three technical replicates each. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

increase was not significantly higher however 10ng/ml of TGF- β 1 induced significant increase of α -SMA expression suggesting that fibroblast to myofibroblast differentiation that was further confirmed by the formation of α -SMA stress fibers, (Figure 3.2C).

3.3.2 High throughput screening of polymer microarray

To study the effect of surface chemistry on fibroblast attachment, morphology, and differentiation an acrylate, methacrylate, and acrylamide library of 300 homo-polymers was contact printed on pHEMA coated glass slides. A full list of homo-polymers library is presented in Appendix 1. The study was separated into two phases: **i)** study of fibroblast attachment and size based at 24 hours of culture and **ii)** study of fibroblast proliferation and α -SMA expression after 96 hours of culture on the polymer microarrays. Following fixation on each time point, the cells were immunostained for cell nuclei (for cell attachment, and for proliferation), F-actin (for cell size) and α -SMA (for fibroblast to myofibroblast differentiation). All results were compared to standard tissue culture plastic (TCP).

3.3.2.1 Polymers influence on fibroblast attachment and size

To investigate fibroblast attachment and size on the homo-polymers the cells were fixed at 24 hours and stained with DAPI to study cell attachment and Phalloidin to study cell morphology. Of the 300 homo-polymer screened most polymers showed fibroblast attachment, (Figure 3.3A). Though, relative to TCP approximately 80 percent of the polymers screened had lower cell attachment. Further observation revealed that based on fibroblast attachment, the homo-

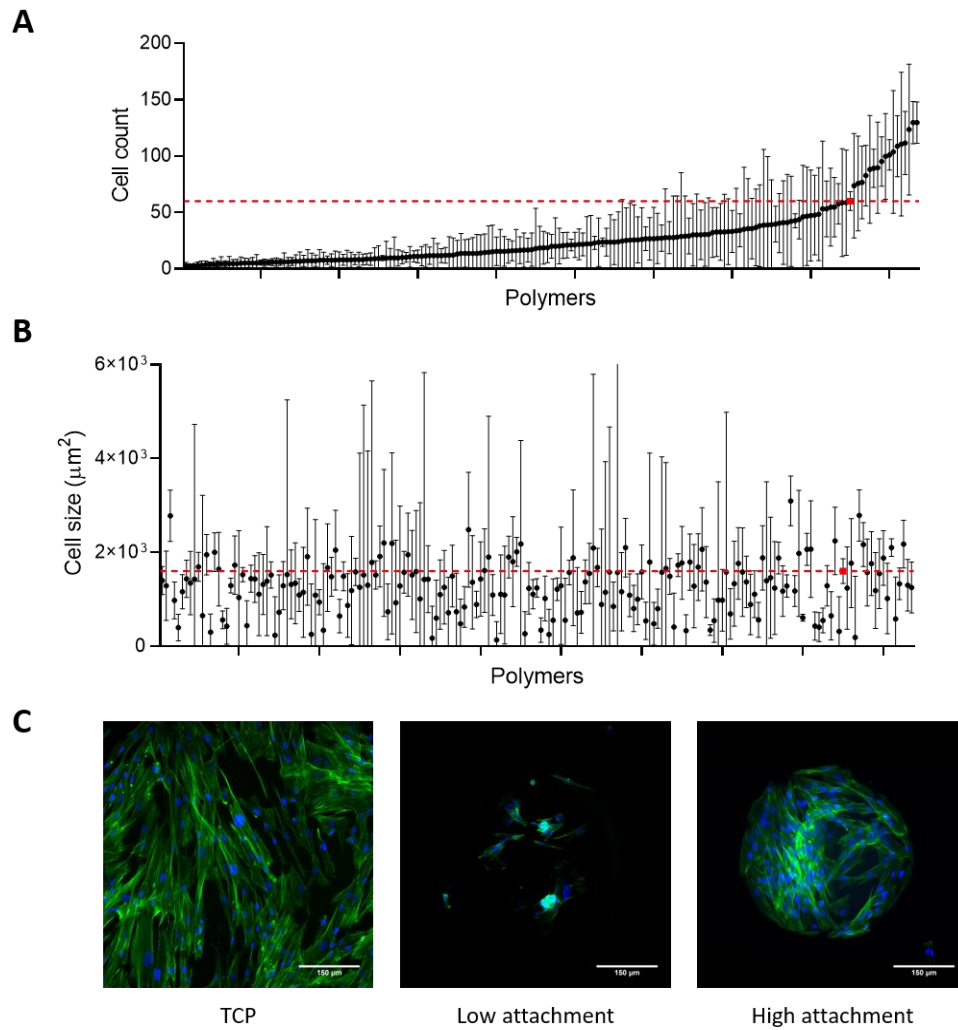


Figure 3.3: High throughput screening data for fibroblast attachment and size A) Rank ordered cell attachment graph. The red line represents TCP. B) Cell size rank ordered with respect to cell attachment. C) Representative images of fibroblasts on polymer spots from 12 collected images. Blue: cell nuclei Green: F-actin. All data shown is from two biological replicates with six technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

polymers could be roughly grouped into; anti-attachment polymers, polymers supporting attachment lower than TCP, polymers attachment equivalent to TCP and polymers enhancing attachment relative to TCP.

Along with cell attachment the other important cell parameter is morphology. The morphology of the cell hints to if the cells has formed strong focal adhesions

with the substrate. To study cell spreading, the cell size (area) was used to identify cells with elongated spindle like morphology or cells which had formed round-like structures. Fibroblasts with smaller cell size are indicative of a lack of spreading thereby hindering cellular functionality [67], [68], [114]. Fibroblasts on the polymers had a wide variety of cell size, (Figure 3.3B). The cell size did not reflect the number of adherent cells and varied across the polymers. This suggested that cell size was not correlated with the number of attached cells. The results imply that though certain polymers promote cell adhesion, these polymers do not necessarily support cell spreading. To identify polymers that supported cell attachment and spreading, a threshold of at least 20 adherent cells (per 300µm diameter polymer spot size) and 60 percent cell size (relative to TCP) was applied, (Figure 3.4A and B). The polymers shortlisted based on this criterion were then assessed against the ‘SNR > 3 criterion’. This enabled removal of data points with high variation. In summary, the methodology enabled selection of polymers that i) supported fibroblast attachment ii) supported cell spreading and iii) had high SNR (low variation).

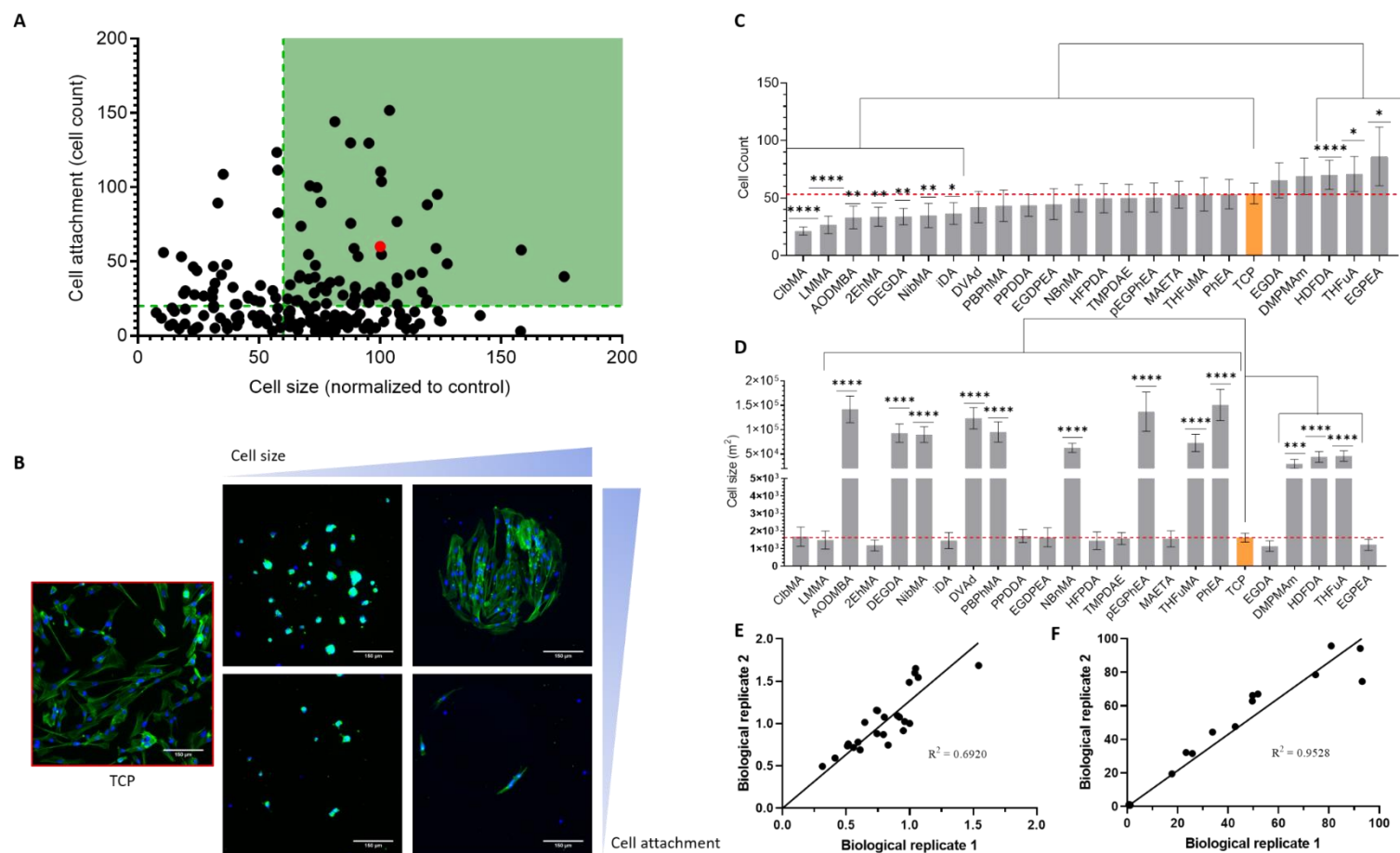


Figure 3.4: Polymer selection based on cell adherence and size. A) Polymer grouping based on fibroblast attachment and size. A cut off 20 adherent cells and 60 percent cell size relative to TCP. All polymers within this green zone were selected for study of functional behaviour. The red spot represents TCP. B) Representative images of cell attachment in relation to cell size. Images are representative of 12 imaged repeats. Blue: cell nuclei Green: F-actin C) Cell attachment and D) cell size of shortlisted polymers based on cell attachment and size criteria. Correlation plots of E) cell size and F) cell attachment between biological replicates. All data shown is from two biological replicates with six technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

A total of 23 polymers were shortlisted based on the fore mentioned criterion (Table A1, Appendix 3) and (Figure 3.4C and D). Poly(ethylene glycol phenyl ethyl acrylate) (pEGPEA), poly(tetrahydrofurfuryl acrylate) (pTHFuA) and poly(heptadecafluorodecyl acrylate) (pHDFDA) were classified as pro-attachment polymers, supporting significantly higher fibroblast attachment relative to TCP. On the other hand, poly((R)- α -Acryloyloxy- β,β -dimethyl- γ -butyrolactone) (pAODMBA), poly(2-ethylhexyl methacrylate)(p2EhMA) and poly(isodecyl acrylate) (piDA) among others, though supported attachment but had significantly lower cell attachment than TCP. An $R^2 = 0.69$ between biological replicates suggested that the polymers influenced cell attachment.

From the 23 shortlisted polymers, 12 polymers had significantly higher mean cellsize compared to TCP. This suggests that these polymers support cell spreading to higher degree, which may in turn influence downstream cellular processes such as cell proliferation and differentiation. The remaining polymers had equivalent cell size to that of TCP. An $R^2 = 0.95$ between the biological replicates suggests that cell size was indeed influenced by the homo-polymers. Following assessment and selection based on cell attachment and morphology the shortlisted polymers were then further studied for fibroblast functional phenotypic behaviour specifically cell proliferation and α -SMA expression.

3.3.2.2 Modulating functional fibroblast behaviour: α -SMA expression and cell proliferation

The functional behaviour of fibroblasts was studied on polymers shown to influence attachment and size. These functional studies were based on observing cell proliferation and fibroblast differentiation into α -SMA expressing myofibroblasts. The fibroblast-polymer interactions were compared to fibroblasts cultured on TCP and, TCP with addition of exogenous TGF- β 1, TCP (+TGF-B1). This pro-fibrotic cytokine is primarily secreted by M2-like macrophages and has been implicated in progressive development of fibrosis [115], [116]. It is known to promote cell proliferation and stimulate differentiation of fibroblasts into myofibroblasts.

Cell proliferation was studied by counting the number of nuclei on the polymers at 24 hours and 96 hours of culture, (Figure 3.5A and B). The ratio of cell count at 96 hours and 24 hours was used to calculate the proliferative index. The pro-fibrotic cytokine TGF- β 1 induced proliferation of cells. This was evident in the fold increase of proliferative index of TCP (1.88) to TCP (+TGF-B1) (2.53), figure 3.5B. The polymers either promoted or suppressed fibroblast proliferation, in comparison to TCP. Polymers such as poly(2-phenylethyl acrylate) (pPhEA), poly(isodecyl acrylate) (piDA), and poly(tetrahydro furfuryl acrylate) (pTHFuA) had significantly higher proliferative index than TCP. A closer observation of these polymers revealed that poly(2-chlorobenzyl methacrylate) (pClbMA), poly(L-menthyl methacrylate) (pLMMA), poly(di(ethylene glycol) diacrylate) (pDEGDA), and poly(isodecyl acrylate) (piDA) had proliferative index greater than TCP (+TGF-B1). This suggested that the forementioned polymers further

impelled fibroblast proliferation in comparison to the growth factor, TGF- β 1. Other polymers such as pAODMBA, poly(4-nitrobenzyl methacrylate) (pNiBMA) and poly(ethylene glycol phenyl ether acrylate) (pEGPEA) actively suppressed proliferation, having an index of 1.60, 1.47 and 1.29, respectively. The phenomena were validated by $R^2 = 0.73$ between first and second biological replicate for proliferation, (Figure 3.5D).

Alongside fibroblast proliferation, the differentiation of fibroblasts into myofibroblasts was studied by measuring the mean fluorescence intensity of myofibroblast marker α -SMA after 96 hours of culture. The myofibroblast phenotype is critical in normal wound healing processes, untimely presence of which has been implicated in fibrotic outcome. Differentiation of fibroblasts and expression of α -SMA is influenced by material stiffness and the pro-fibrotic growth factor, TGF- β 1. The addition of exogenous TGF- β 1 stimulated fibroblasts on TCP (+TGF-B1) to express significantly higher mean intensity of α -SMA, implicating the growth factor in the differentiation process. Fibroblasts cultured on the polymers induced higher mean intensity of α -SMA, with exclusion of p(NibMA), p(THFuA) and p(iDA), which induced lower mean intensity of α -SMA. The increase in intensity may be due to the higher stiffness of the polymers. Moreover, fibroblasts cultured on poly(pentabromophenyl methacrylate) (pPBPhMA), poly(4-methacryloxyethyl trimellitic anhydride) (pMAETA), and poly(n-[3-(dimethylamino)propyl]methacrylamide) (pDMPMAm) enhanced mean intensity of α -SMA in comparison to TCP (+TGF-B1). This suggested that these polymers promoted a greater fibrotic

outcome than the outcome produced by the effect of pro-fibrotic, TGF- β 1. The observation was validated with an $R^2 = 0.99$ between the two biological replicates, (Figure 3.5E).

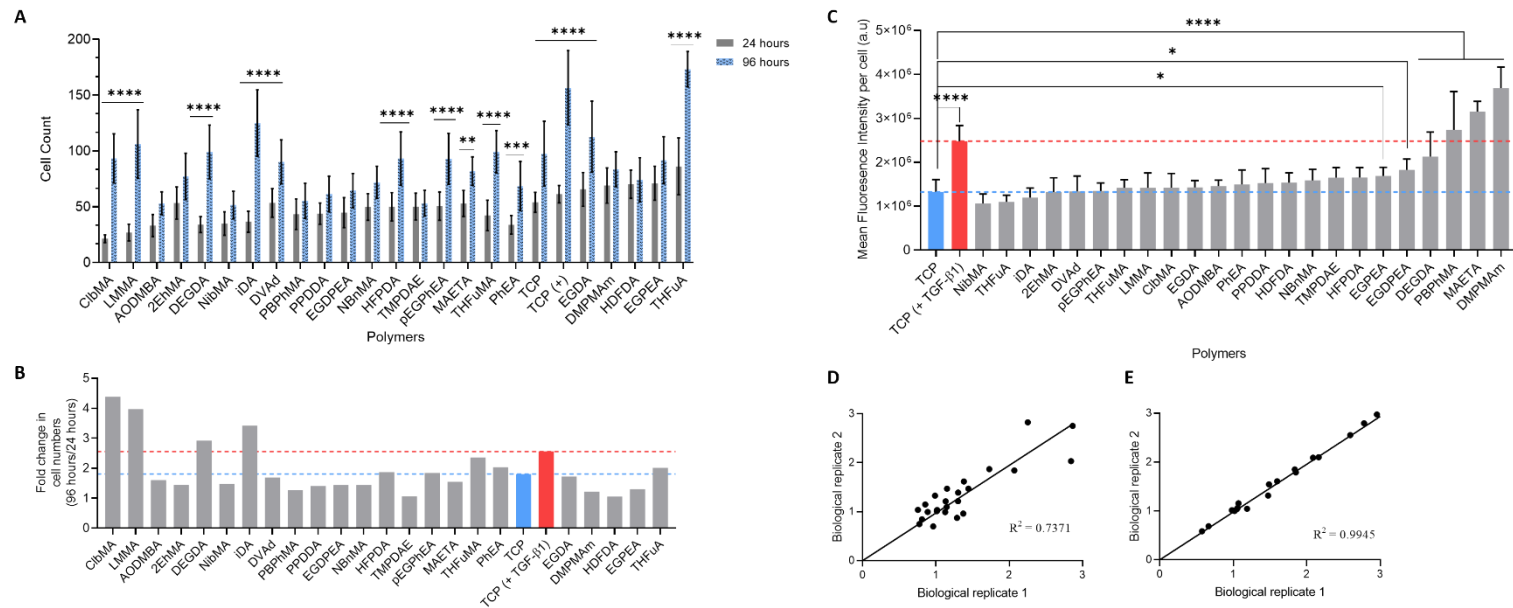


Figure 3.5: The functional behaviour of fibroblasts was studied on the polymers by measuring cell proliferation and expression of α -SMA A) Cell proliferation was measured by counting the number of adherent cells at 24 hours and 96 hours of culture B) The proliferative was calculated based on the ratio of cell count at 96 hours and 24 hours of culture. The blue bar represents proliferative index if cells cultured on TCP and red bar represents cells cultured on TCP with pro-fibrotic growth factor C) The mean fluorescence intensity of α -SMA after 96 hours of culture. The blue bar represents intensity of cells on TCP and red bar intensity of cells on TCP with pro-fibrotic growth factor D) Correlation plot between biological replicates for cell proliferation. E) Correlation plot between biological replicates for MFI of α -SMA. All data shown is from two biological replicates with six technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.2.3 Polymer selection for scaled-up studies

To better understand the effects of the polymers on fibroblast proliferation and differentiation, the fold change in proliferative index and fluorescence intensity of α -SMA (with respect to TCP) was plotted, (Figure 3.6). This enabled visualization of the modulatory ability of the polymers relative to TCP control. Based on this understanding, the polymers were classified into four groups. Polymers that **i)** increase in cell proliferation and decrease in intensity of α -SMA (+ CP – SMA) **ii)** increase in cell proliferation and decrease in intensity of α -SMA (+ CP + SMA) **iii)** decrease in cell proliferation and decrease in intensity of α -SMA expression (- CP – SMA) **iv)** decrease in cell proliferation and increase

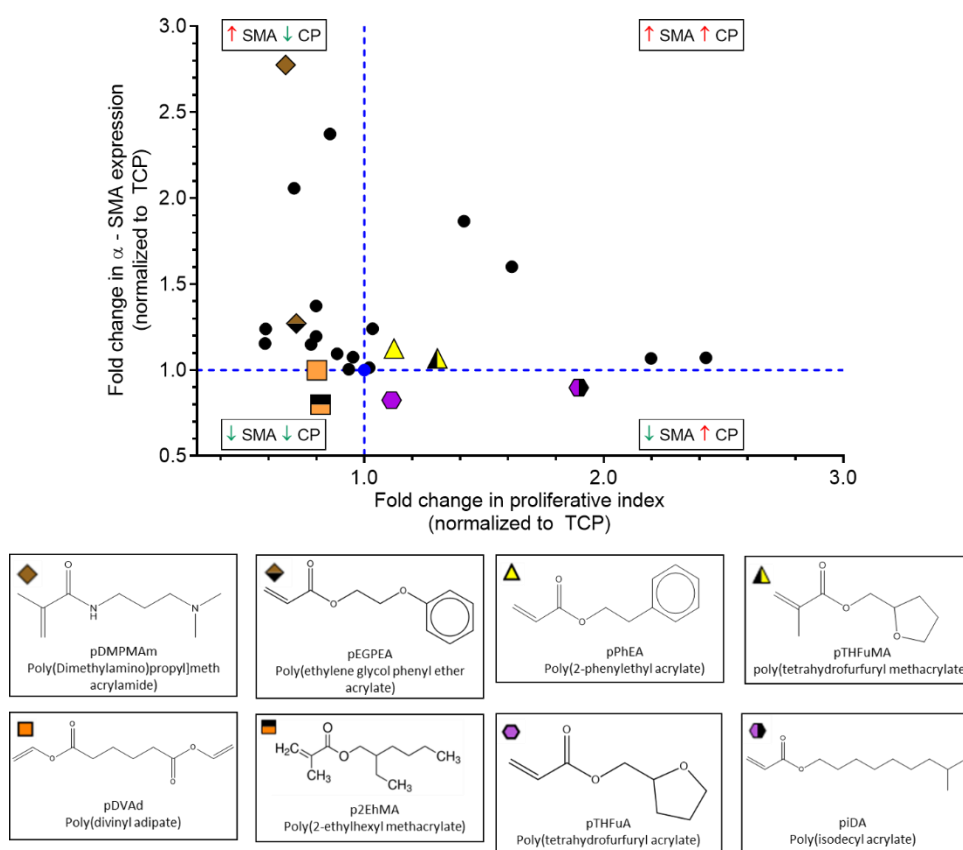


Figure 3.6: **Polymer grouping of homo-polymers for hit selection.** The polymers could be grouped into four based on their modulatory behaviour. Two polymers with the lowest COV from each quadrant were selected for scaled up studies.

in intensity of α -SMA expression (- CP + SMA). Using this classification, polymers modulating fibroblast α -SMA expression and/or proliferation could be selected. To perform additional characterisation and functional studies, two polymers from each group with lowest coefficient of variation (COV) were selected to be scaled up.

3.4 Discussion

The implantation of medical devices induces a host response which results in chronic inflammation and may lead to formation of a fibrous capsule around the device [58]. This acellular fibrous capsule does not mimic repaired tissue and limits functionality of the medical device; ultimately leading towards its failure. Stromal cells particularly fibroblasts have been directly implicated in the development of this fibrotic capsule, though these cells are vital for normal wound repair and regeneration [15], [109], [110]. This presents an opportunity to directly modulate the formation of the fibrous capsule by controlling fibroblast phenotype at the biomaterial interface.

In this study, a high throughput screening methodology was applied to discover polymers that could influence fibroblast behaviour: such as adhesion, size, proliferation, and differentiation to myofibroblasts. Primarily surfaces should be able to support fibroblast attachment and spreading via integrin engagement, for other downstream cellular processes such as proliferation or differentiation to occur. Differential fibroblast attachment was observed on the polymer substrates; with polymers such as poly(hydroxy butyl acrylate) (pHBA) and poly(hydroxy ethyl acrylate) (pHEA) acting as anti-attachment surfaces and pEGPEA as pro-

attachment surfaces. The difference in adherence could be explained through the selective protein adsorption on polymers. Numerous studies have observed that protein adsorption is necessary for cell attachment where low protein adsorption induces a lack of cell attachment [92], [93], [117], [118]. Specifically, the adsorption of fibronectin and vitronectin favours fibroblast attachment. This suggests that fibroblast attachment is not only influenced by protein adsorption but also the conformation and type of protein adsorbed. These studies suggest that the screened polymers may have differential protein adsorption as well as selective adsorption of ECM components which then subsequently influence fibroblast attachment.

The area covered by the cell, termed cell size, was used as the marker for cell spreading [67], [118], [119]. The measure was normalized against cell number, this allowed comparison between polymers with varying degrees of adherent cells. A dramatic range of cell size was observed across the polymers, which did not correlate with the cell attachment; suggesting that cell attachment did not influence cell spreading. For instance, though pEGPEA had significantly higher cell attachment than TCP, it had equivalent cell size. On the other hand, pAODMBA had significantly lower cell attachment than TCP, and it had significantly higher cell size than TCP; even though cell spreading is concomitant result of cell attachment and has similar biomaterial surface characteristics governing it. As with cell attachment, fibronectin and vitronectin have been delineated as ECM adsorbates that support cell spreading. Both fibronectin and vitronectin present ligands which engage integrin receptors to

form focal adhesions, thus supporting cell spreading. Polymer surface characteristics such as hydrophilicity and hydrophobicity influence adsorption of the ECM adsorbates as well as their conformations; resulting in poor or high cell spreading. Specifically, studies have shown that cell spreading is supported more on moderately hydrophilic and hydrophilic/anionic surfaces than on hydrophobic and non-ionic hydrophilic surfaces [67]. Thus, the differences in cell attachment and spreading on polymer surfaces may be attributed to differential adsorption and conformation of ECM adsorbates due to polymer surface characteristics. Furthermore, studies have shown that negatively charged cell membrane adhere closely to positively charged surfaces, indicative of higher cell attachment [28]. Though this surface charge influences initial cell attachment, the spreading and downstream cellular functions require engagement of integrins to the adsorbed proteins. Thus, a positive surface charged polymer may support cell attachment but due to lack of adsorbed proteins it would not encourage cell spreading. The adherent fibroblasts should not only be able to adhere to the polymer surfaces but should also be able to adopt a cell morphology like that of fibroblasts cultured on TCP, thus mimicking *in vivo*.

The stark differences in cell attachment and size hinted at the need for investigating cell attachment in conjunction with cell size. A qualitative assessment was used to determine a cut off for cell size, below which cells exhibited rounded morphology indicative of poor cell spreading. Similarly, a cut-off of 20 cells per 300µm diameter spot for cell attachment was determined based on qualitative assessment. Homo-polymers within this cut-off were then assessed

on the $SNR > 3$ criterion. The SNR method removed polymers with high variance. In summary, polymers that supported reasonable cell attachment and spreading, and had low variation were shortlisted. The initial attachment and spreading of cells are vital for viability and downstream cellular processes. Moreover, cell spreading has been linked in governing downstream cellular processes such as proliferation and differentiation to myofibroblasts [28], [67], [118]. Both are important phenotypic traits in the context of wound healing. Thus, the selection of candidate polymers that supported reasonable cell attachment and proliferation could possibly direct downstream cellular processes.

The proliferative and remodelling phases of wound healing consists of proliferating fibroblasts, neo-expression of ECM components and fibroblast differentiation into α -SMA positive myofibroblasts. Biomaterial implantation dysregulates these processes resulting in impaired wound healing [15], [22], [25]. Following so, the effect of homo-polymers on cell proliferation and expression of myofibroblast marker α -SMA was assessed. As stated earlier, cell proliferation was measured by calculating a proliferative index (ratio between cell count at 96 hours and 24 hours). The pro-fibrotic growth factor, TGF- β 1, is known to be secreted by M2-like macrophages and is crucial for normal wound healing [58], [59]. Though it has also been implicated in furthering fibrotic potential of fibroblasts. In this study, the inclusion of TGF- β 1 enabled the assessment of modulatory ability of the polymers in comparison to a fibrotic microenvironment, TCP (+TGF-B1). The proliferative index of the polymers

varied with polymers either promoting or suppressing fibroblast proliferation, in comparison to TCP. Specifically, polymers such as pPhEA, pTHFuA and piDA enhanced proliferation compared to TCP. Other polymers suppressed proliferation, pAODMBA and pEGPEA. It was noted that certain polymers with high proliferative potential, such as pTHFuA, also supported higher cell attachment compared to low attachment, low proliferative index polymers, such as pNibMA and pAODMBA. It is well established that ligand-integrin binding during cell attachment and spreading impacts the proliferation of cells. Furthermore, the reorganization of surface-adsorbed fibronectin is an essential requirement for cell proliferation [28], [120]. The latter compromised on hydrophobic surfaces, thereby limiting the proliferative potential of cells. This suggests that a pro-attachment polymer may have a lower proliferative index if it hindered reorganization of surface-adsorbed fibronectin. The converse being a low attachment polymer facilitating reorganization of surface-bound fibronectin, thus augmenting proliferation. Moreover, studies have noted that the adsorbed fibronectin may undergo material-driven fibrillogenesis, thus enhancing its bioactivity [121], [122]. This material-driven process may rationalize the ‘super’ proliferative nature of fibroblasts observed on polymers, such as piDA and pLMMA, having proliferative index higher than TCP (+TGF-B1).

In conjunction with cell proliferation the differentiation of fibroblasts to myofibroblasts was studied by measuring the mean fluorescence intensity (MFI) of α -SMA. The excessive and persistent presence of myofibroblasts has been linked with a fibrotic outcome. Fibroblast differentiation to myofibroblasts is

influenced by stiffness of the biomaterial surface/ECM microenvironment and by TGF- β 1 mediated signalling. A dose dependent study of TGF- β 1 showed that a concentration of 10ng ml⁻¹ for 96 hours significantly increased the fluorescence intensity of α -SMA of fibroblasts on cultured TCP [55], [67], [68]. As with the proliferation study, the expression of α -SMA was additionally compared to a fibrotic condition, TCP (+TGF-B1). The MFI of α -SMA of fibroblasts on polymers could be grouped into polymers that enhanced α -SMA expression or suppressed α -SMA expression relative to TCP. For example, pDMPMAm and pMAETA had higher intensity while pTHFuA and piDA had lower α -SMA intensity, relative to TCP. These findings suggest that pDMPMAm and pMAETA would support excessive differentiation and presence of myofibroblasts, where as pTHFuA and piDA would limit differentiation. Substrate stiffness has been shown to regulate fibroblast differentiation to myofibroblasts, thus regulating α -SMA expression [123]–[125]. Therefore, it is reasonable to hypothesise that polymers that induced a higher intensity α -SMA may have a higher substrate stiffness than other polymers and TCP, thus furthering expression of α -SMA. Moreover, studies have shown that stiffness is more potent than TGF- β 1 mediated differentiation processes to myofibroblasts; thus, explaining the higher intensity levels observed with cell cultured on pPBPhMA, pMAETA and, pDMPMAm relative to the fibrotic condition, TCP (+TGF-B1). Another explanation is the modulation of bioactivity of adsorbed fibronectin by material-driven fibrillogenesis, which would drive excessive differentiation (expression of α -SMA), as was the case with modulation of cell proliferation.

To better understand the overall functional effects of the polymers on fibroblast behaviour, the fold changes (with respect to TCP) for cell proliferation and MFI of α -SMA were plotted against each other. With TCP as the reference point, the polymers could then be grouped into four groups based on their ability to modulate proliferation and α -SMA expression: each quadrant having its own characteristic applicability. For example, in the case of surgical meshes for hernia repair or pelvic floor reconstruction, excessive production of ECM components is desired to help hold the mesh in place post-surgery. Polymers that promote cell proliferation and myofibroblast presence may be coated onto the mesh, which could help increase both cell number and ECM deposition. To further study and characterise fibroblast phenotype and secretions, two polymers (with the lowest COV) representative of each phenotype quadrant were selected for polymerisation and scaled up studies.

In summary, the high throughput screening methodology applied in this study proved effective in identifying polymers with ability to modulate key functional properties of fibroblasts. The ‘hit’ polymers selected from the screening supported cell attachment and spreading, in addition to modulating fibroblast proliferation and differentiation to myofibroblasts. Further, these ‘hit’ polymers were selected for additional characterisation and functional studies.

Chapter 4: Development and characterization of polymer coated substrates

Chapter 4 - Development and characterisation of polymer coated substrates

4.1 Introduction

Synthetic polymers, specifically (meth)acrylates and acrylamides, are increasingly used in dentistry, orthopaedics, ocular and neuro-prosthetics as inert fillers and coatings [126]–[128]. For instance, poly(methyl methacrylate) (PMMA) is inert, nondegradable and has a refractive index of 1.5 making it the standard implant material for intraocular lenses [129]. These polymers offer a degree of control over chemical and mechanical properties, degradation rates and can be moulded into different structures, making them viable and attractive for medical applications.

Polymeric biomaterials have an abundance of functional groups including hydroxyl, amino, and carboxylic acid groups [130]. These functional groups, in conjunction with initiators or crosslinkers, can be utilized to polymerize and crosslink these biomaterials, which can then form hydrogels, such as Gelatin meth acryloyl (GelMA), or be cast as polymer films, such as polyurethane films.

Free radical polymerisation (FRP) is a method of polymer synthesis that can be easily applied to a large set of monomers and requires moderate synthesis conditions, in comparison to other polymerisation methods [130], [131]. Depending on the type of initiator, FRP can either be UV driven (using a photochemical initiator) or thermal driven (using a thermal initiator). Thermal FRP uses an initiator which generates a pair of free radicals in response to heat. These free radicals react with the functional groups polymerizing the monomer.

The reaction produces a batch of polymer that can then be solubilized and cast onto various surfaces and substrates, making it a versatile FRP method. In contrast to UV driven FRP where individual surfaces require to be coated and UV irradiated. For this reason, thermal FRP was chosen as the polymerisation method.

Polymeric films can be fabricated by either drop casting or spin coating polymer solutions onto substrates. Both methodologies produce thin polymer films, though spin coating provides a greater degree of control over thickness and uniformity of the films, while ensuring a hole-free film [100], [101]. More so, the technique has been consistently used to fabricate acrylate and acrylamide films to study cell behaviour *in vitro* and *in vivo* [122], [132], [133]. Time-of-flight secondary ion mass spectroscopy (ToF-SIMS) is a surface sensitive technique used to characterise and analyse material surfaces [102]. The technique has been used to analyse biomaterial surfaces for uniform hole-free polymer coating by studying the distribution of characteristic polymer ion fragments [88], [134], [135].

Biomaterial surfaces have been studied to instruct cell behaviour via two routes i) direct, by varying surface topography and stiffness which targets cellular mechano-sensing and ii) indirect, by influencing adsorption and conformation of serum proteins through surface properties such as the wettability, and charge. There are various surface characterisation techniques that have been developed to study biomaterial surface characteristics, such as measuring the Young's modulus for material stiffness, the water contact angle to measure wettability,

and X-ray photoelectron spectroscopy (XPS) to measure thickness of the bio-adsorbate layer[103], [136]. The physical stiffness of biomaterials has been studied to instruct cell differentiation: increased fibroblast differentiation to myofibroblasts has been observed on stiffer hydrogels and thin films[123], [137], [138]. The water contact angle has been used to inform of the hydrophilicity or hydrophobicity of material surfaces; which is known to effect protein adsorption onto the surface, which subsequently influences cell behaviour[78], [107]. XPS is a surface sensitive technique used for quantitative measurement of elemental composition of substrates. In the context of characterising biomaterial surfaces, XPS has been used to estimate the thickness of adsorbed proteins on surfaces by measuring elemental nitrogen present in adsorbed proteins[105], [111].

In the previous chapter, the high throughput screening of an acrylate, methacrylate and acrylamide library for fibroblast modulation revealed eight hit homopolymers that supported cell attachment, and modulated proliferation and differentiation. This chapter discusses thermal FRP and characterisation of the hit polymers, coating onto glass coverslips, and subsequent surface characterisation. This methodology forms the base for further studying fibroblast-polymer interactions in subsequent chapters.

4.2 Materials and methods

4.2.1 Polymer synthesis

All chemicals were purchased from Sigma Aldrich, Scientific Polymers or Polysciences and used as supplied without purification. In brief, 2g of monomer, thermal initiator Azobisisobutyronitrile (AIBN) and free radical transfer catalyst

bis[(difluoroboryl) diphenylglyoximato]cobalt(II) (COPhBF) were solubilized in dry toluene at given concentrations (table 4.1). The reaction mixture was sealed and degassed for 1 hour, prior to initiation at 85°C. The reaction was then terminated after 18-24 hours. The resultant polymers were isolated by gently pouring them into an excess of n-hexane, resolubilized in dichloromethane and re-precipitated in excess n-hexane. This procedure was repeated thrice after which the isolated polymers were dried under vacuum for 7 days prior to use. The polymerisation conditions for each polymer were optimized using a trial-and-error methodology and varying the concentrations AIBN and COPhBF.

4.2.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H NMR spectra were recorded at 25°C using a Bruker DPX-300 spectrometer (400 MHz). Chemical shifts were recorded in δH (ppm). Samples were dissolved in deuterated chloroform (CDCl_3) to which chemical shifts are referenced (residual chloroform at 7.26 ppm).

4.2.3 Gel Permeation Chromatography Analysis (GPC)

GPC analysis was performed using an Agilent 1260 infinity instrument. The measurement was performed using THF as the mobile phase with a flow rate of 1 mL min^{-1} at 25°C. The GPC samples were prepared in HPLC grade THF and filtered with $0.22\mu\text{m}$ filter prior to injection. Analysis was performed using the Astra software to calculate number average molecular weight (M_n), polydispersity (D) and the degree of polymerisation.

4.2.4 Polymer casting onto coverslips

Polymers (2% w/v in solvent) were spin coated at 2000rpm for a given time using a spin coater (Mk7, Cordell group) on 13mm glass coverslips. The coated surfaces were dried in a vacuum oven at for 4 days and placed in distilled water at 37°C for a further 2 days, before sterilization with UV light.

4.2.5 ToF-SIMS

ToF-SIMS measurements were carried out with a TOF.SIMS IV instrument (IONTOF GmbH, Muenster, Germany) equipped with a bismuth cluster primary ion source (Bi_3^+ , 25 keV), as previously described[88]. Data analysis of the measurements were carried out with the IonToF software (IONTOF GmbH).

4.2.6 Water contact angle measurements

The water contact angle (WCA) of the polymer films was measured using a CAM 200 contact angle apparatus (KVS). The WCA was measured by pipetting 5 μl of ultrapure water on each polymer and measuring the angle that forms between the water drop and the polymer surface. All measurements were repeated four times for each polymer.

4.2.7 X-ray photoelectron spectroscopy (XPS) and estimation of protein thickness

The polymer films were either treated with serum-free cell culture medium (untreated) or with serum-containing medium (FBS/MEM) for 24 hours. The samples were rinsed with ultrapure water thrice to remove unbound proteins prior to the XPS measurements. The measurements were carried out using the Kratos AXIS ULTRA with a monochromated aluminium $\text{K}\alpha$ X-ray source (1486.6eV)

as previously described[88]. The spectra on both, untreated and FBS/MEM treated polymer films, were acquired with the Kratos VISION II software. The wide scan was used to identify elements present on the surface of the polymer films. CasaXPS software (Version 2.3.19) was used to analyse spectra generated from the wide scans. The elemental compositions (in atomic percentages) of carbon (C), oxygen (O), and nitrogen (N) were quantified by measuring the elemental peak areas on the acquired spectra.

The adsorbed protein thickness on polymer films of untreated and FBS/MEM treated polymers was quantified by the following equation[105]:

$$d_{N(1s)} = -L_{N(1s)} \cos \theta \left(1 - \frac{[N] - [N]_0}{[N]_{\infty} - [N]_0} \right)$$

Where L = electron attenuation length (3.02nm), $\cos(\theta) = 1$, [N] = measured nitrogen fraction on FBS/MEM treated polymer substrate, $[N]_{\infty}$ = atomic fraction of nitrogen in serum (15%) and $[N]_0$ = nitrogen fraction on untreated polymer films.

4.2.8 Atomic force microscopy (AFM)

The stiffness measurements using the Atomic Force Microscope (AFM) was measured by collaborators at the School of Pharmacy. The MFP-3D Standalone AFM (Oxford Instruments, Asylum Research Inc., CA) was used to obtain force-displacement curves of the polymer samples in water for Young's modulus calculation. Following data collection, the Derjaguin-Muller-Toporov mathematical model was used to fit the slope of the retracting curve using least squares regression line for calculation of Young's modulus.

4.3 Results

4.3.1 Polymer synthesis and analysis

A total of eight of homo-polymers identified from the chapter 3 were synthesised by thermal free radical polymerisation. A trial-and-error approach was used to determine successful polymerisation parameters for each polymer, this approach is summarised in figure 4.1. Polymerisation was influenced by change in parameters such as AIBN %, CPhBF ppm and reaction times. All eight

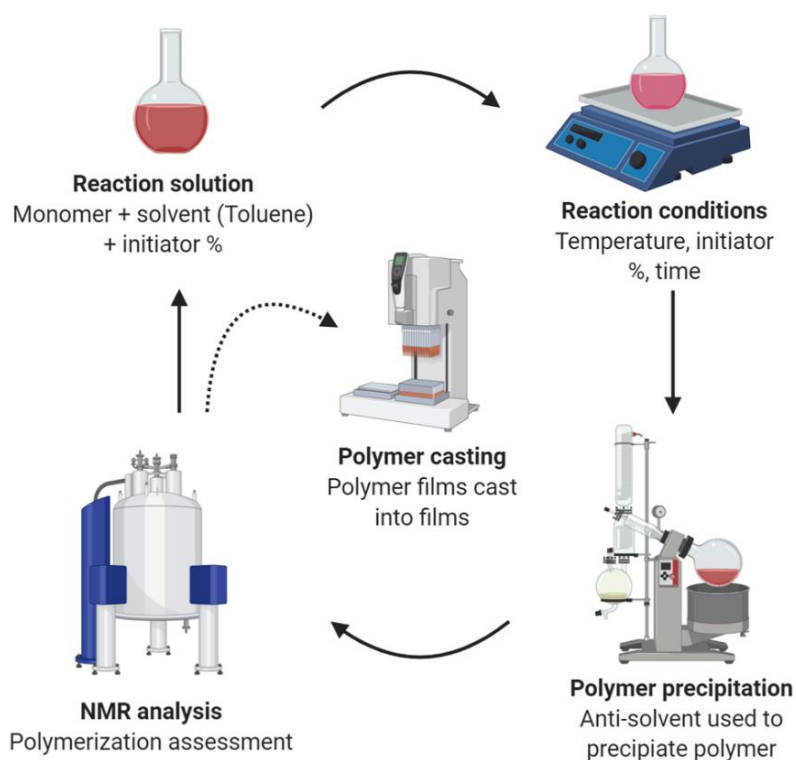
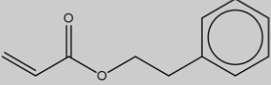
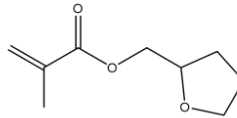
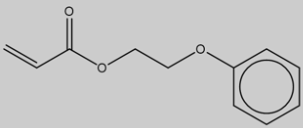
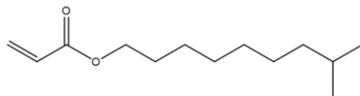
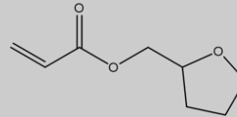
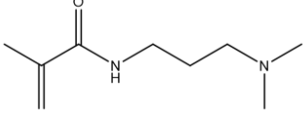
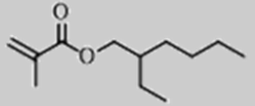
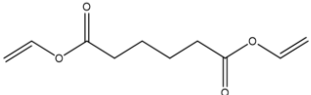


Figure 4.1: Hit monomers were polymerised by thermal free radical polymerisation and polymerisation assessed with NMR analysis. In brief, this involved a trial-and-error process of polymer synthesis in reaction conditions, precipitating the synthesised polymer in anti-solvent and assessing polymerisation with NMR analysis. The successfully synthesised polymers were then spin coated on to 13mm round glass coverslips.

monomers were successfully polymerised, and their reaction parameters are summarised in table 4.1.

Table 4.1: Reaction parameters used to polymerize hit polymers

MONOMER	STRUCTURE	AIBN (wt/v%)	COPhBF (ppm)
Phenyl Ethyl Acrylate (PhEA)		0.5	-
Tetrahydrofurfuryl metacrylate (THFuMA)		0.5	-
Ethylene glycol phenyl ether methacrylate (EGPEA)		0.5	850
Isodecyl acrylate (IDA)		0.5	-
Tetrahydrofurfuryl acrylate (THFuA)		0.5	-
N-[3-(Dimethylamino)propyl]metha crylamide (DMPMAm)		0.5	50
2-ethylhexyl methacrylate (2EhMA)		0.5	-
Divinyl adipate (DVAd)		0.5	250

After reaction termination, successful polymerisation was crudely determined by assessing whether reaction mixture was viscous and if the mixture successfully precipitated in an anti-solvent. Reaction mixtures that had solidified were considered cross-linked and increasing concentrations of CPhBF were tested in attempt to limit rate of reaction. Successful polymerisation was accurately

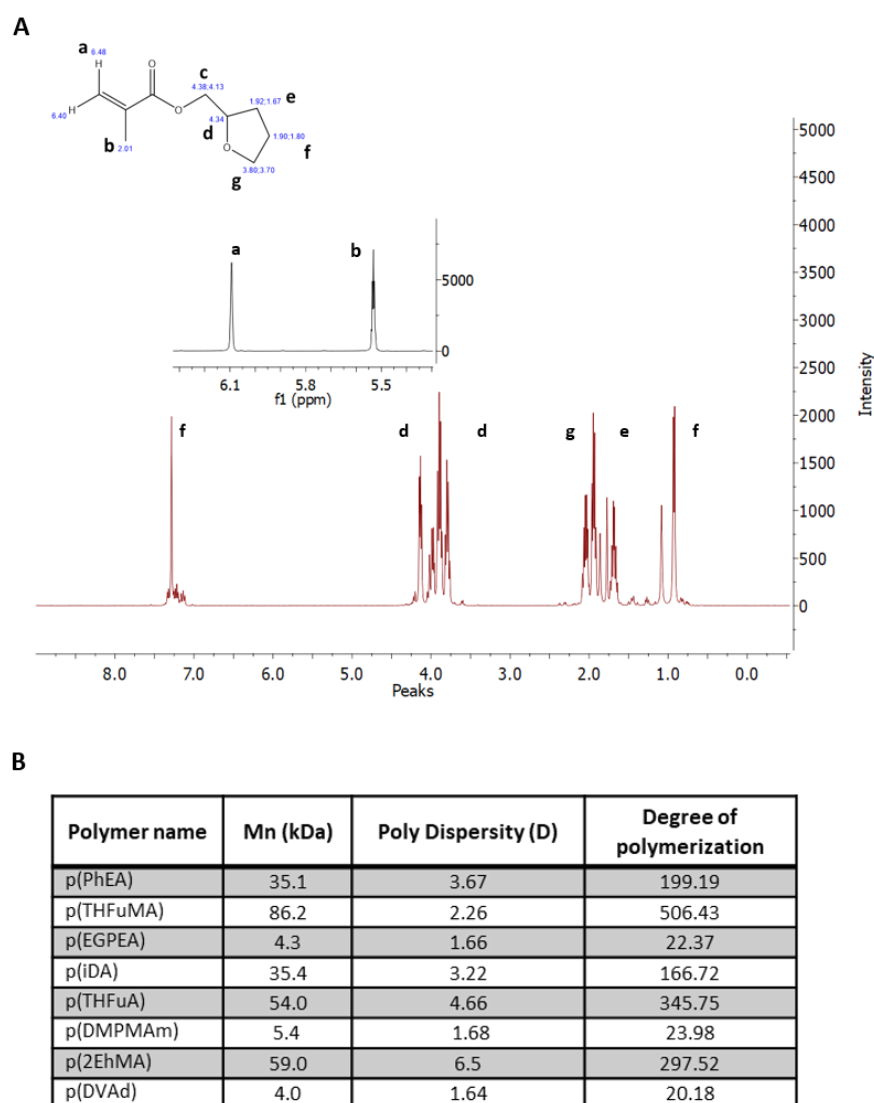


Figure 4.2: **Characterisation of synthesised polymers** A) The ^1H NMR spectra for p(THFuMA) with the polymer spectra in red and the peaks for the methacrylate functional group shown black. The absence of peaks for the functional group in the polymer spectra indicate successful polymerisation. B) Polymer characteristics such as molecular weight, poly dispersity index and degree of polymerisation were measured/calculated.

assessed by studying the ^1H NMR spectra of the polymer and its monomer, (Figure 4.2A). The presence or absence of the two proton peaks for the (meth)acrylate group found between 5.0 and 7.0 is a clear indicator of successful polymerisation and/or presence of trace monomer. The ^1H NMR spectra for all synthesised polymers are presented in figure A5 of Appendix 4.

The synthesised polymers were then further characterised by measuring the M_n , calculating the polydispersity, and degree of polymerisation. These results are summarised in figure 4.2B. All polymers had an M_n greater than 30kDa with exception of p(EGPEA), p(DVAd), and p(DMPMAM). Similarly, the polydispersity for all polymers was greater than 2.5 with exception of p(EGPEA), p(DVAd), and p(DMPMAM).

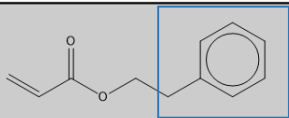
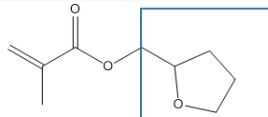
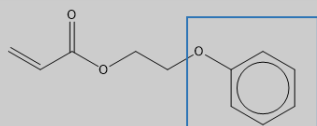
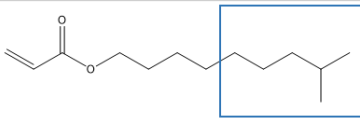
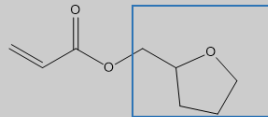
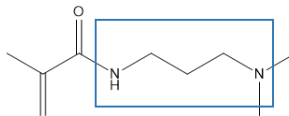
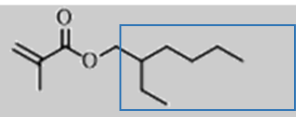
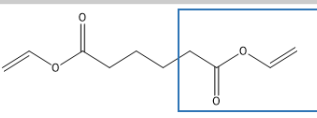
4.3.2 Spin coating and characterisation

The synthesised polymers were solubilized in solvent at 2% (w/v) and spin coated onto glass coverslips. The polymer solution was pipetted onto the coverslip and spun for a specific spin time, figure 4.3A summarizes this process. A trial-and-error approach was used to optimize spin duration for each polymer. Spin times were assessed by visually inspecting coverslips for complete coating. The spin times for each polymer are summarised in figure 4.3B.

The ToF-SIMS surface analysis was used to assess uniformity of polymer films coated on the coverslips. Both positive and negative ion spectra were acquired for each polymer film. The coating was assessed by identifying unique ion identifiers for each polymer film, (table 4.2). For example, the pTHFuMA film coating was assessed by observing the $\text{C}_5\text{H}_9\text{O}^+$ ion on uncoated glass coverslips

and on pTHFuMA coated coverslips, (Figure 4.3C). It was noted that the polymer was uniformly coated on the coverslips, as was evident by the ion distribution. The ToF-SIMS spectra for all polymer coated coverslips is presented in figure A4 in Appendix 4.

Table 4.2: Identifier ion fragments for ToF-SIMS analysis

<u>POLYMER</u>	<u>POLYMER STRUCTURE</u>	<u>IDENTIFIER ION</u>
Poly(phenyl ethyl acrylate) p(PhEA)		$C_7H_7^-$
Poly(tetrahydrofurfuryl methacrylate) p(THFuMA)		$C_5H_9O^+$
Poly(ethylene glycol phenyl ether methacrylate) p(EGPEA)		$C_6H_5O^-$
Poly(isodecyl acrylate) p(iDA)		$C_6H_{13}^+$
Poly(tetrahydrofurfuryl acrylate) p(THFuA)		$C_5H_9O^+$
Poly(N-[3-(Dimethylamino)propyl]methacrylamide) p(DMPMAm)		$C_3H_7N_2^-$
Poly(2-ethylhexyl methacrylate) p(2EhMA)		$C_5H_{11}^+$
Poly(divinyl adipate) p(DVAd)		$C_3H_3O_2^-$

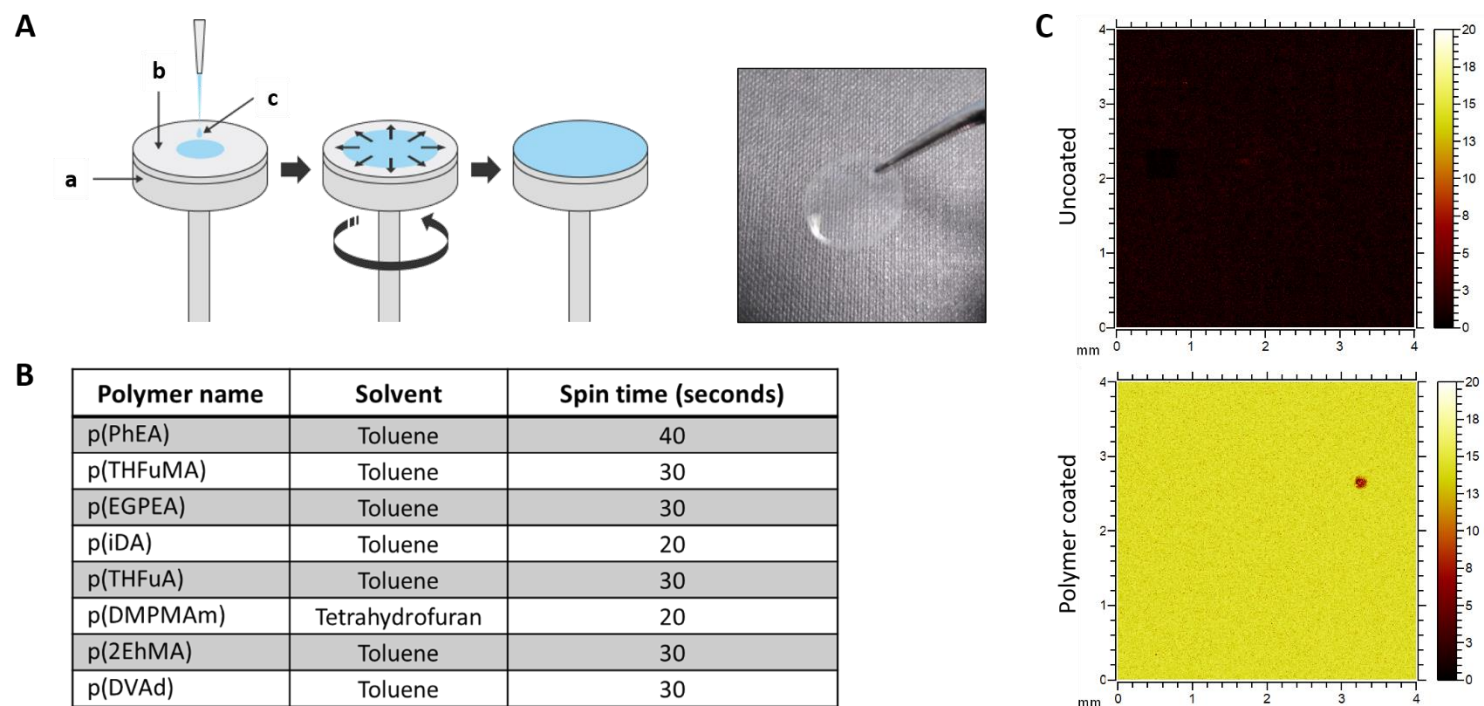


Figure 4.3: **Polymers were spin coated onto glass coverslips and coating assessed using ToF-SIMS.** A) Spin coating involves three components a) a rotating base b) coating substrate such as coverslips and c) the polymer solution which is pipetted onto the rotating substrate. The polymer solutions is spread uniformly across the substrate with the centripetal force that is generated by the rotating base. This results in a uniformly coated polymer film as shown. B) The eight homopolymers were solubilised in volatile solvent and the optimized spin time for each polymer is shown. C) ToF-SIMS spectra images for the $C_5H_9O^+$ ion present on pTHFuMA shows that the polymer was uniformly coated onto the glass coverslips.

4.3.3 Surface and physical characterisation of polymer films

The surface properties of the polymer films were characterised by studying the water contact angle (WCA) and the stiffness of the polymer films (in water) was measured using AFM, (Figure 4.4A and B). The highest WCA was observed on piDA with a mean value of 108° which was significantly higher than WCA of pDVAd and TCP, both having similar angles with a mean value of 66° and 62°, (Figure 4.4B). The pDMPMAM film had the lowest WCA with a mean value of 20°. Based on surface hydrophilicity the polymer could be ordered as follows: piDA < p2EhMA < pPhEA < pEGPEA < pTHFuA < pTHFuMA < pDVAd < TCP < pDMPMAM. To study the relationship between molecular composition of the polymer and measured WCA, the elemental composition of carbon (C) and oxygen (O) of polymers was determined by analysing the polymer structures. Polymer O:C ratios were subsequently calculated. A strong negative correlation was observed between the elemental O:C ratio and the measured WCA, (Figure 4.4C). Polymers with high elemental oxygen had lower WCA, such as pDMPMAM, whereas polymers with low elemental oxygen had higher WCA, such as p2EhMA.

Further characterisation involved studying material stiffness of the polymers using an atomic force microscope. The highest stiffness was observed on p2EhMA having a mean modulus of 4.7GPa followed by pDVAd with 3.6GPa, and TCP with 2.6GPa, (Figure 4.4A). The following polymers had similar mean modulus that ranged between 1.0GPa and 1.7GPa: pPhEA, pTHFuMA, pTHFuA, piDA, pEGPEA and pDMPMAM. In comparison to TCP, these

polymers had significantly lower stiffness whereas the p2EhMA film had significantly higher stiffness. These results demonstrated that the polymer films had differentiating surface properties, in particular the WCA, which in-turn may influence the cell behaviour on these films.

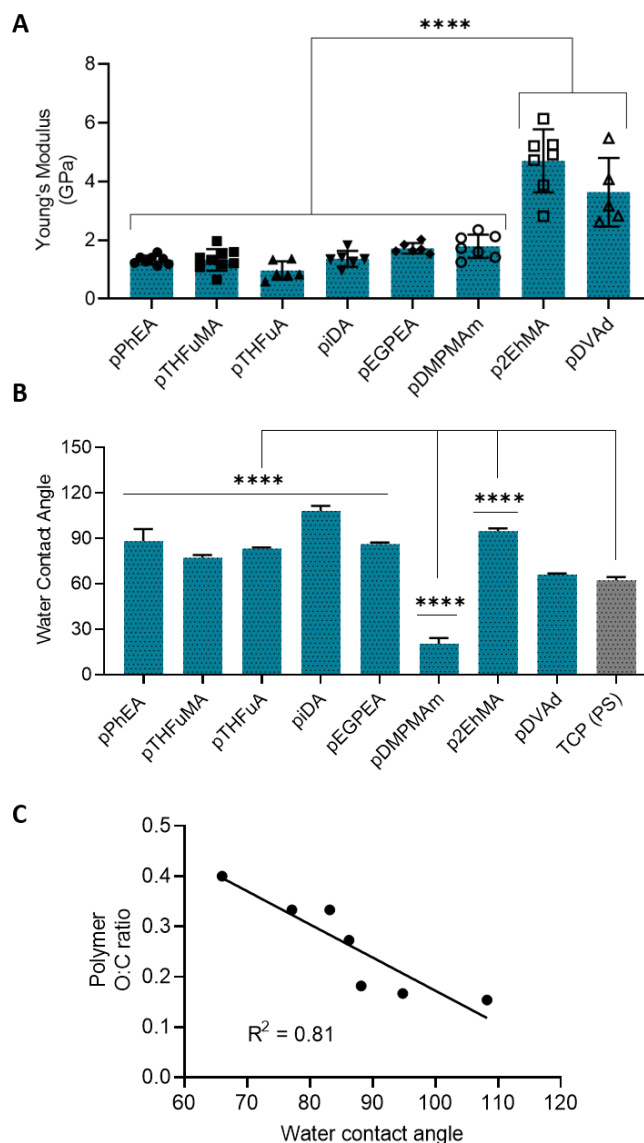


Figure 4.4: Surface characterisation of polymer coated coverslips. A) The stiffness of the polymer films was measured by studying the Young's modulus acquired using an AFM B) Water contact angle measurements for polymer surfaces. TCP – Tissue culture plastic (polystyrene) C) Correlation of polymer WCA measurements with O:C ratio. All data shown is from i) 16 measurement each for stiffness and ii) two independent measurements with four technical replicates each for water contact angle measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.3.4 Bio-adsorbate thickness on polymers

To investigate the influence of polymer chemistry on adsorption of serum proteins, the polymers were treated with serum containing culture medium (FBS/MEM) or with serum-free culture medium (untreated), as previously described. The polymers were then analysed using XPS, and the atomic fraction of nitrogen present on untreated and FBS/MEM treated films measured. The measured nitrogen fraction was indicative of the adsorbed protein layer [105]. The spectra acquired for untreated pTHFuMA films had peaks for carbon (C1s) and oxygen (O1s), which were constituent for the polymer, and had no detected peaks for nitrogen (N1s), (Figure 4.5A). Following treatment with serum containing medium (FBS/MEM), a nitrogen peak (N1s) was observed suggesting that proteins had adsorbed onto the polymer surface. All polymer surfaces treated with FBS/MEM exhibited varying levels of adsorbed proteins, as was shown by the measured nitrogen fractions, (Figure 4.5B). It was noted that elemental nitrogen was detected on untreated pDMPMam films, which were attributed to the nitrogen in the tertiary amine of the polymer.

The measured nitrogen fraction on the polymers were applied to calculate the thickness of adsorbed proteins following FBS/MEM treatment. The thickest protein over-layer on the polymers was observed on pPhEA and pDMPMam, having 3.19nm and 3.09nm respectively, (Figure 4.5C). Whereas the lowest protein over-layer was measured on pTHFuA and piDA with 0.8nm and 0.55nm, respectively, which was 4-fold lower than the highest estimated protein thickness. The pTHFuMA, pEGPEA and pDVAd films had similar protein

thickness with 2.20 nm, 2.18 nm, and 2.95 nm. In comparison to TCP, all polymer films had significantly lower adsorbed protein over-layer. These results suggested that polymers such as pPhEA actively promoted protein adsorption while surfaces such as pTHFuA and piDA impeded adsorption of serum proteins.

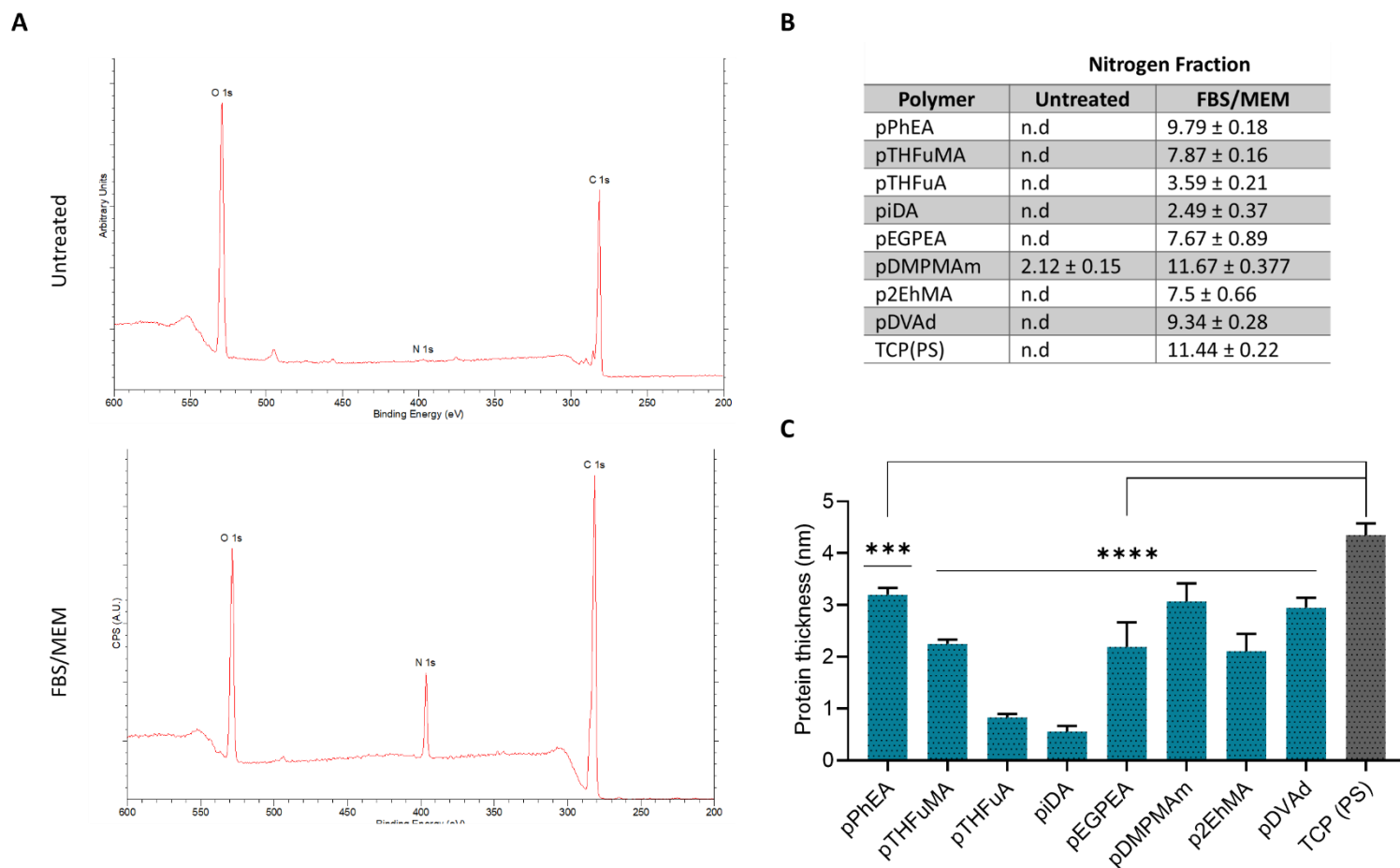


Figure 4.5: Analysis of adsorbed protein thickness on polymer films. A) XPS spectra of pTHFuMA films following untreated and FBS/MEM treatment. The peaks for O1s, N1s and C1s are marked. B) The measured elemental nitrogen detected on polymer films from the XPS spectra. All data shown is mean ± standard deviation ($n = 3$) C) The elemental nitrogen fraction was used to calculate the estimated adsorbed protein thickness on polymer films. All data shown is from two independent measurements with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.4 Discussion

Synthetic polymers are increasingly used in the medical industry for a variety of applications such as dental fillers, orthopaedic coatings, cardiovascular stents, and wound dressings. These polymers possess physiochemical properties that make them ideal for specific applications. For instance, polyurethane is extensively used in vascular grafts, stents, and other soft tissue devices. Its glass transition temperature (T_g) is less than room temperature giving it an elasticity, that closely matches that of blood vessels [129]. Though these polymers are biocompatible they are known to induce acute inflammation, which may lead to an FBR.

In the previous chapter meth(acrylate) and acrylamide homopolymers were identified that modulated fibroblast attachment, size, proliferation, and differentiation. To further study these hit polymers they were synthesised using thermal FRP and cast onto glass coverslips.

There are several polymer syntheses routes in addition to traditional FRP, such as anionic polymerisation and controlled/living polymerisation. These different syntheses routes offer the ability to control polymer properties, such as molecular weight, polydispersity, and chain architecture [130], [139], [140]. Though these syntheses routes offer better control over polymer properties, conventional FRP has distinct advantages. The most important being that FRP is versatile and can polymerize a large library of acrylate and acrylamide monomers due to the non-specific nature of free radicals towards the acrylate and acrylamide functional

groups [130]. This was advantageous as it limited optimization required for synthesis of the eight hit homopolymers.

In contrast to the UV FRP used to polymerize monomers on the polymer microarray, the polymers for the scaled-up studies were synthesised using thermal FRP. The thermal FRP allowed a batch of polymer to be synthesised in comparison to UV FRP, where monomers were polymerised on substrates *in situ*. This reduced the batch-to-batch variation with UV FRP substrates. Additionally, the thermal FRP polymers could be coated onto different geometrical substrates (for example membranes or scaffolds).

All eight hit polymers were successfully synthesised from their monomers using thermal FRP. A trial-and-error approach was used to optimize the synthesis conditions for each polymer. All polymers were synthesised with AIBN 0.5 % (w/w) and the reaction was initiated at 85°C. These conditions allowed a similar rate of chain propagation resulting in high molecular weight polymers, as was observed from molecular weights of the polymers, with exception of p(EGPEA), p(DVAd), and p(DMPMAm). In reactions where the polymer crosslinked, in the cases of p(EGPEA), p(DVAd), and p(DMPMAm), a chain transfer catalyst CPhBF was added. The chain transfer catalyst acts to ‘slow down’ reaction kinetics by reacting with a growing polymer chain to yield terminated species [141]. This addition not only resulted in successful polymerisation but as expected lowered the molecular weights of the polymers, as is for p(EGPEA), p(DVAd), and p(DMPMAm).

The degree of polymerisation measures the number of monomeric units in a polymer molecule. It is calculated as the ratio between the molecular weight of the polymer and the molecular weight of the monomer, the latter forming the repeat unit in the polymer chain [142]. All eight synthesised polymer had a degree of polymerisation above 100, with exception of p(EGPEA), p(DVAd), and p(DMPMAm). This was expected as the synthesised polymers had high molecular weight, while the chain transfer catalyst lowered the molecular weight of p(EGPEA), p(DVAd), and p(DMPMAm) thus lowering their degree of polymerisation. The degree of polymerisation effects the mechanical tensile strength of polymers, where a higher degree of polymerisation results in an increase in tensile strength [143], [144]. This may indicate that polymers with higher degree of polymerisation may have higher tensile strength as compared to polymers with lower degree of polymerisation.

The polydispersity of polymers informs of the distribution of polymer chains in the polymer. For polymers that have monodisperse chains, such as proteins, the polydispersity value is 1.0. For synthetic polymers, a polydispersity value ranging from 1.5 to 2.0 is regarded as acceptable. The polydispersity for the synthesised polymers varied, suggesting that they had a varying chain distribution. This was expected as FRP offers limited control over polydispersity. The addition of the chain transfer catalyst provided a degree of control, which is evident in polydispersity values for p(EGPEA), p(DVAd), and p(DMPMAm).

The synthesised polymers were coated on glass coverslips by spin coating a 2% (w/v) polymer solution, as previously described [131], [132]. The poly(acrylate)

and poly(methacrylate) coated substrates were analysed with ToF-SIMS and assessed for uniform, hole-free coating. This was evaluated by identifying unique ion fragments specific for each polymer detected on the coated substrates. The ToF-SIMS image spectra had strong identifier ion fragment signals present on the coated coverslips compared to the uncoated coverslips. These observations concluded that spin coating methodology produced uniform polymer films on glass coverslips.

The mechanical properties of the coated polymers were studied by measuring stiffness using an AFM. The results obtained showed that all polymers had a similar stiffness range, with exception of p2EhMA and pDVAd which had higher stiffness. An increase in mechanical stiffness of polymers has been associated with an increase in molecular weight of the polymer [145]. Interestingly, the range of molecular weights measured for the synthesised poly(acrylate)s and poly(methacrylate)s was not mirrored by the range of stiffness measured. As aforementioned, the spin coating technique offers a degree of control over the thickness of the produced films, by controlling parameters such as rotation speed, time, and viscosity of the polymer solution [100], [101]. The polymer films were fabricated with similar coating parameters which suggested that the films had similar thickness, thus elucidating the narrow range in measured stiffness. The decrease in viscosity of p2EhMA and pDVAd solutions compared to other polymer solutions may form thin films, which elucidates the increase in measured stiffness.

Further surface characterisation involved measuring the wettability of the polymer films by measuring the water contact angle. According to Vogler's definition, surfaces having water contact angles less than 65° are classified as hydrophilic [146]. It was observed that most polymer films had hydrophobic characteristics with the piDA film having the highest water contact angle, 108° . The comparator, TCP, had a slightly hydrophilic surface similar to the pDVAd film. The DMPMAm film had the most hydrophilic surface with a water contact angle of 20° . The variation in the measured wettability can be associated with the surface roughness and the atomic oxygen content of the polymer films [107], [147]. A correlation plot between the O/C ratio (atomic oxygen content) and the water contact angle showed that the O/C ratio of the polymers negatively influenced the water contact angle measurements for all polymers, excluding pDMPMAm. This suggested that an increase in the oxygen content of the polymers resulted in a decrease of the water contact angle, leading to a less hydrophobic surface. A similar trend was observed with oxygen etched polystyrene films [107]. The decrease in the water contact angle with decrease atomic oxygen content for the pDMPMAm film suggested that surface roughness instead of atomic oxygen content may be its driving factor.

Cells interact with polymeric biomaterials by engaging with the adsorbed protein layer. Thus, by studying protein adsorption the behaviour of cells may be explicated. XPS analysis was used to study the thickness of the bio-adsorbate layer on untreated and FBS/MEM treated polymer films. The detection of elemental nitrogen on the FBS/MEM treated polymers revealed the presence of

adsorbed proteins following treatment. The intensity of the measured elemental nitrogen from the polymers was used to determine the thickness of the protein over-layer [105]. Protein adsorption onto biomaterial surfaces is dependent on surface properties such as wettability, charge, polarity, and topography. Studies have outlined that hydrophobic surfaces favour adsorption of bulk proteins from solutions. Conversely, other studies have reported higher levels of adsorbed proteins on hydrophilic surfaces compared to hydrophobic surfaces. Interestingly, the hydrophobic, pPhEA and piDA films, had the highest and lowest estimated protein thickness, respectively. These results suggested that protein adsorption on these films may be guided by other surface characteristics such as roughness and charge.

In summary, the hit polymers identified from chapter 3 were successfully synthesised using thermal FRP and analytically characterised. Furthermore, the synthesised polymers were coated onto glass coverslips, and were shown to form uniformly coated films using ToF-SIMS analysis. The polymer films were shown to have a range of stiffness and wettability properties. Further, the XPS analysis showed that the surface properties could influence protein adsorption, which may then modulate cell behaviour. In conclusion, these scaled up polymers and coatings served as foundation to study in-depth fibroblast behaviour on the hit polymers.

Chapter 5: Controlling stromal cell behaviour using bio-instructive surfaces

Chapter 5 – Controlling stromal cell behaviour using bio-instructive surfaces

5.1 Introduction

Fibroblasts are stromal cells found within the ECM of the connective tissues. These cells have well defined roles in homeostasis of the ECM, inflammation, angiogenesis, cancer progression, as well as in wound healing and fibrosis. Fibroblasts play a pivotal role in wound healing and fibrosis, where in response to chemical factors secreted by macrophages and/or ECM stiffness of the microenvironment the cells differentiate to α -SMA expressing myofibroblasts and upregulate secretion of collagens [2], [15], [23].

In normal wound healing, fibroblasts (and myofibroblasts) deposit structural ECM that replaces the provisional fibrin matrix. This is mostly composed of basket-weave like collagen III, which in the later stages of wound healing is replaced by highly ordered collagen I. The presence of implanted biomaterials derail normal wound healing processes causing upregulated fibroblast differentiation to myofibroblasts which forms collagen rich acellular fibrous capsule around the implanted biomaterial. The formation of this fibrous capsule has been linked with persistent presence of myofibroblasts. Furthermore, studies have indicated that a higher collagen III to collagen I ratio at the wound site is indicative of prolonged inflammation and thicker fibrous capsule formation[42], [44], [148], [149]. Though fibroblasts and myofibroblasts are necessary for effective repair and resolution of healing wounds, a disbalance in the cellular microenvironment can lead to overstimulation of myofibroblasts which results

in generation of a fibrous capsule, such as the case in biomaterial mediated wounds (implanted medical devices).

The two dominant cell types in the wound healing microenvironment are macrophages and fibroblasts (including myofibroblasts) [150], [151]. Macrophages are immune cells that in response to chemical stimuli and biomaterial surface properties polarise into pro-inflammatory, M1-like and/or anti-inflammatory, M2-like macrophages. Each of these subsets express distinct surface markers and secrete classifiable concentrations of cytokines and growth factors. Notably, M1-like macrophages secrete pro-inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 and IL-12. Whereas M2-like macrophages secrete anti-inflammatory cytokines such as IL-10, CCL18, IL-1RA and $\text{TGF-}\beta$ 1. The latter known as a pro-fibrotic cytokine, promoting fibroblast proliferation and differentiation into myofibroblasts.

Fibroblasts themselves secrete both autocrine and paracrine signalling cytokines and chemokines, such as bFGF, $\text{TGF-}\beta$ 1, HGF, IL-6 and MCP-1. These secreted molecules further influence macrophages, fibroblasts, and endothelial cells in the microenvironment. For example, bFGF promotes fibroblasts and endothelial cells to proliferate while MCP-1 creates a chemotaxis gradient for circulating monocytes to the wound site. Topical application or release of bFGF and MCP-1 from scaffolds has been shown to promote wound healing in chronic wounds [64], [152]–[154]. However, these strategies were proven to be unsustainable due to their short half-life [64]. These findings imply that biomaterials must be

designed such that they can not only modulate fibroblast phenotype and behaviour but also modulate their secretory profile.

In the previous chapter, polymers which supported fibroblast attachment and spreading while modulating proliferation and differentiation were identified. These were classified into four phenotypic groups: **i)** promoting cell proliferation and suppressing differentiation to myofibroblasts (+ CP – SMA) **ii)** promoting both cell proliferation and differentiation (+ CP + SMA) **iii)** suppressing cell proliferation both cell proliferation and differentiation (- CP – SMA) and **iv)** suppressing cell proliferation and promoting differentiation (- CP + SMA). This chapter would validate the findings of the previous chapter, and furthermore study the influence of polymers on fibroblast secretome, specifically cytokines, growth factors and ECM components. Additionally, the impact of the polymers on fibroblast-mediated wound closure would be studied. Lastly, the mechanism driving polymer-cell interactions would be explored.

5.2 Materials and methods

5.2.1 Cell culture

The human lung fibroblasts (CCL-171, ATCC) and human skin fibroblasts (CRL-2522, ATCC) were cultured in Minimum Essential Medium Eagles (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, non-essential amino acids, penicillin/streptomycin, and sodium pyruvate (Sigma). The cells were cultured in T75 flasks at 37°C with 5% supplemental CO₂ until 90% confluent before passaging. For pro-fibrotic culture controls, fibroblasts were cultured with recombinant human TGF-β1 (10 ng ml⁻¹) (R & D systems) for 96 hours on TCP.

5.2.2 Cytotoxicity

The cell viability of fibroblasts on the polymer films was assessed using the ToxiLight™ non-destructive cytotoxicity bioassay kit (Lonza) at 24 hours of culture. The assay measures the release of adenylate kinase (AK) from cells with compromised cell membranes. The AK catalyses conversion of ADP to ATP, which is then detected through bioluminescence. The emitted light intensity is linearly related to the AK concentration, and can be detected using a plate reader.

5.2.3 Immunostaining

5.2.3.1 Cell attachment and proliferation

The cells were fixed and permeabilized with 0.15% triton-x (Sigma) after 24 hours of culture. To probe for cytoskeleton the cells were incubated with Alexa Fluor® 647 Phalloidin 1:50 dilution (Cell Signalling Technology) for 30 minutes. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Cell proliferation was measured with the Baseclick EdU imaging kit (Baseclick). EdU was added to cell medium at 3μM and at 72 hours of culture, where it was incorporated into newly synthesised DNA during the G1/S phase of the cell cycle. After 24 hours, the cells were fixed and stained for EdU following manufacturer's instructions.

5.2.3.2 Measuring MFI of α – smooth muscle actin (SMA)

At 96 hours of culture, the cells were fixed and permeabilized with 0.5% triton-x (Sigma). Non-specific binding proteins were blocked with 10% goat serum for 30 minutes. The cells were targeted for anti α -smooth muscle actin 5μg/ml (clone 1A4, Sigma) and an Alexa Fluor® 546 5μg/ml (Thermofisher Scientific) secondary

antibody. Fluorescence intensity was quantified by measuring the integrated density of α - smooth muscle actin positive cells, this was normalized against the total number of cells.

5.2.4 ELISA

The concentrations of bFGF, HGF, IL-6, and MCP-1 secreted into the medium by fibroblasts cultured on polymer films was analysed after 96 hours of culture with Duoset ELISA kits (R&D Systems) according to manufacturer's instructions.

5.2.5 RT-PCR

Total RNA was isolated from cells using RNeasy plus kit (Qiagen) followed by cDNA synthesis using a qPCRBIO cDNA synthesis kit (PCR Biosystems). Real-time PCR was performed on the MxPro 3005P qRT-PCR system (Stratagene) using 2x qPCRBIO SyGreen Blue mix Lo-Rox (PCR Biosystems), according to manufacturer's instructions. Forward and reverse primers were purchased from Eurofins Scientific UK and Sigma, these are listed in table 5.1. Gene expression levels were first normalised to housekeeping gene, glyceraldehyde-3-phosphate

Table 5.1: Table of primers used to study fibroblast phenotype using an RT-PCR.

Genes	Primer	Sequence (5'-3')
GAPDH	Forward	ACAGTCCATGCCATCACTGCC
	Reverse	GCCTGCTTCACCACCTTCTTG
SMA	Forward	AGACCCTGTTCCAGCCATC
	Reverse	TGCTAGGGCCGTGATCTC
Collagen I (α 1)	Forward	GTCGCACTGGTGATGCTG
	Reverse	GGTGGTGTCCACCTCGAG
Collagen III (α 1)	Forward	AGCTGAAAAGAGTGGTGACAG
	Reverse	CCTTGAGGACCAGGAGCAC

dehydrogenase (GAPDH), and then the relative expression levels were calculated with the $2^{-\Delta\text{ct}}$ method.

5.2.6 Wound healing assay

In vitro wound assays were performed using IBIDI self-culture inserts as previously described [155], [156]. The IBIDI self-culture inserts were used to form wells on polymer films (pTHFuA, pTHFuMA, pEGPEA and pDVAd). Briefly, when confluent layer of human skin fibroblasts (BJ) was formed, the insert was removed, and cells washed twice with PBS. The wounded monolayers were then cultured in complete medium for up to 96 hours. The samples were fixed and stained with CellMask Green 2 µg/ml (Invitrogen), and imaged.

5.2.7 Imaging and image analysis

Mounted samples were imaged using the Zeiss TIRF (Carl Zeiss) device in widefield mode. Image analysis routines to measure cell attachment, proliferation, MFI of α -SMA and wound area were developed using FIJI ImageJ. Details of these routines are presented in appendix 2.

5.2.8 Data and statistical analysis

All data acquired from polymer films is expressed as mean $\pm$ standard deviation, with two independent biological replicates and three technical replicates each. Statistical significance was calculated using one-way or two-way ANOVA and the Tukey's post hoc analysis, whereby $p \leq 0.05$ was considered as being statistically significant.

5.3 Results

5.3.1 Polymer films influence fibroblast attachment and morphology

Both Human lung fibroblasts (MRC-5) and skin fibroblasts (BJ) were cultured on polymer films for 24 hours prior to measurement of Adenylate Kinase (AK) levels, to assess cytotoxicity. Both cell types had low levels of AK in their supernatant suggesting high cell viability on the polymer films, (Figure 5.1A).

In addition to cell viability, the cells were fluorescently labelled and imaged for nuclei and cytoskeleton (F-actin). Cell attachment was studied by counting the number of nuclei, which related to the number of adherent cells. The cell attachment profile of MRC5 fibroblasts on the polymer films positively correlated with the cell attachment profile of the fibroblasts on the polymer microarray, having a $R^2 = 0.75$, (Figure 5.1D). Moreover, the cell attachment of MRC-5 fibroblasts cultured on polymer films positively correlated with cell attachment profile of BJ fibroblasts cultured on polymer films, having a $R^2 = 0.89$, (Figure 5E). These results suggested that biological efficacy of homopolymers identified in the high throughput screen was conserved in the scale up studies. More so, the polymer films had similar influences on attachment of fibroblasts originating from different tissues.

The BJ fibroblasts had significantly lower cell attachment on all 8 polymer films compared to the TCP control, with exception of pEGPEA which had an attachment profile equivalent to TCP control, (Figure 5.1B). The attachment profile on the polymer films varied with pEGPEA having highest cell number

with approximately 200 cells attached per FOV, and with pTHFuA having lowest cell attachment with approximately 100 cells attached per FOV.

In addition to attachment the polymer films effected fibroblast morphology, (Figure 5.1F). Fibroblast morphology was studied by segmenting cytoskeleton using image analysis routines, and measuring morphometric parameters: aspect ratio, average size, circularity and perimeter, (Figure A6 in Appendix 4). To interpret cell morphology, the aspect ratio of fibroblasts was plotted against average size, (Figure 5.1C). Fibroblast morphology highly varied on the polymer films, as evident by the large error bars. Based on cell morphology the fibroblasts could be roughly grouped into **i)** cells with low aspect ratio and high average size, characterised by an approximate aspect ratio of 2.6 and average size of $3000\mu\text{m}^2$ **ii)** cells with high aspect ratio and low average size, characterised by an approximate aspect ratio of 3.0 and average size of $2000\mu\text{m}^2$. This suggested that the cells either adopted a spread-polygonal-like morphology or an elongated spindle-like morphology, (Figure 5.1E).

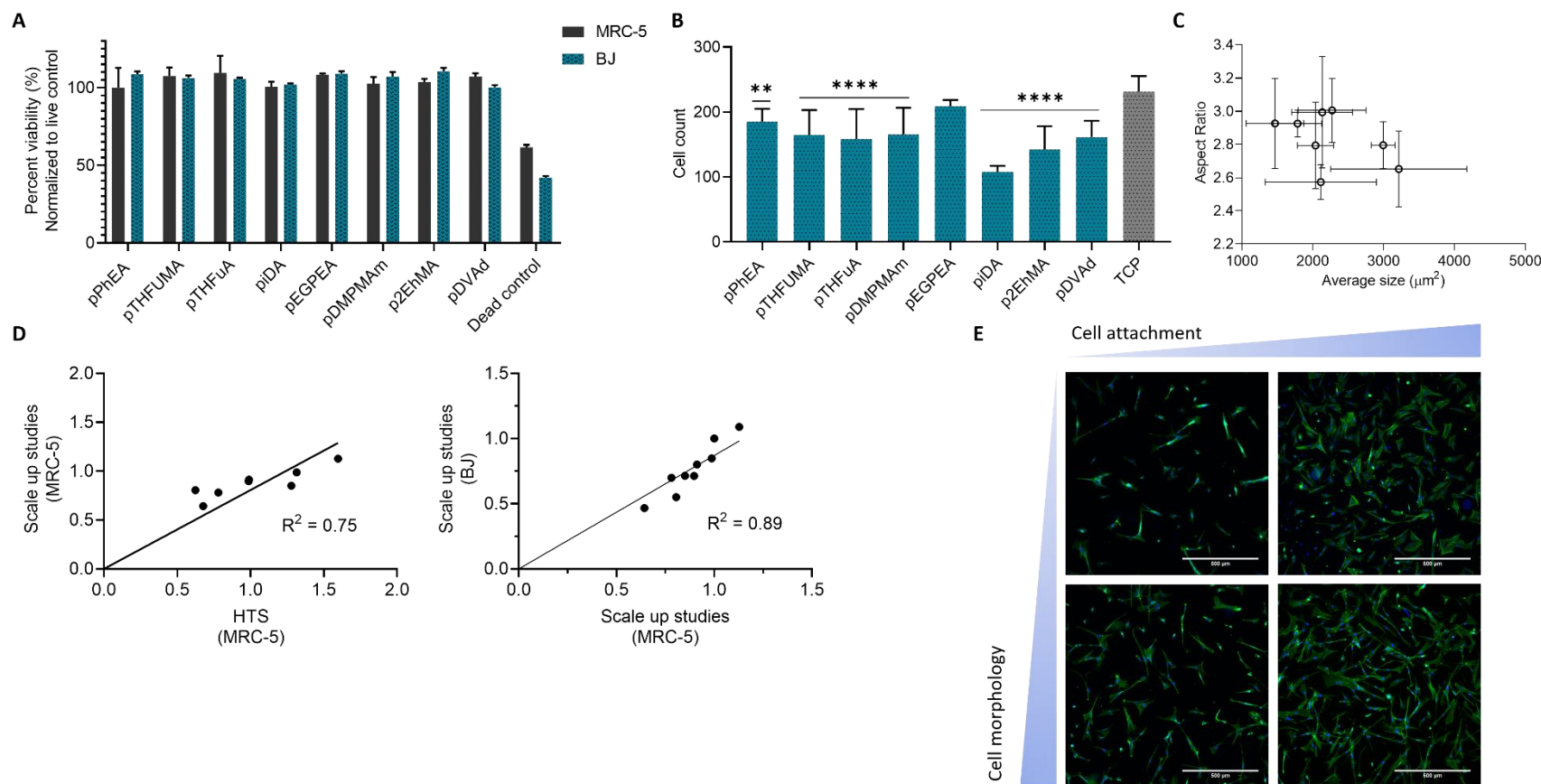


Figure 5.1: Fibroblast cytotoxicity, attachment, and morphology on scaled-up polymers. The fibroblasts were cultured on spin coated polymer films for 24 hours before assessment for A) Cytotoxicity. The cells were shown to have high cell viability on all eight polymer films B) Cell attachment. The fibroblasts had differential levels of cell numbers across the polymer films. C) Cell morphology. Fibroblast aspect ratio was plotted against average size. D) Correlation plots between scale up studies and high throughput studies, and between skin and lung fibroblasts on scaled up polymers E) Representative images of fibroblasts showing cell attachment and morphology. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.2 Modulation of fibroblast phenotype

Fibroblasts cultured on polymer films were studied for modulation of phenotypic traits, cell proliferation and expression of myofibroblast marker α -SMA, after 96 hours of culture. Cell proliferation was studied by measuring the incorporation of EdU into newly synthesised DNA. The detected EdU positive cells were in the G1/S phase of the cell cycle and thus indicated proliferating cells. An additional pro-fibrotic control included fibroblasts cultured on TCP with exogenously added TGF- β 1, TCP (+TGF-B1); this simulated a pro-fibrotic

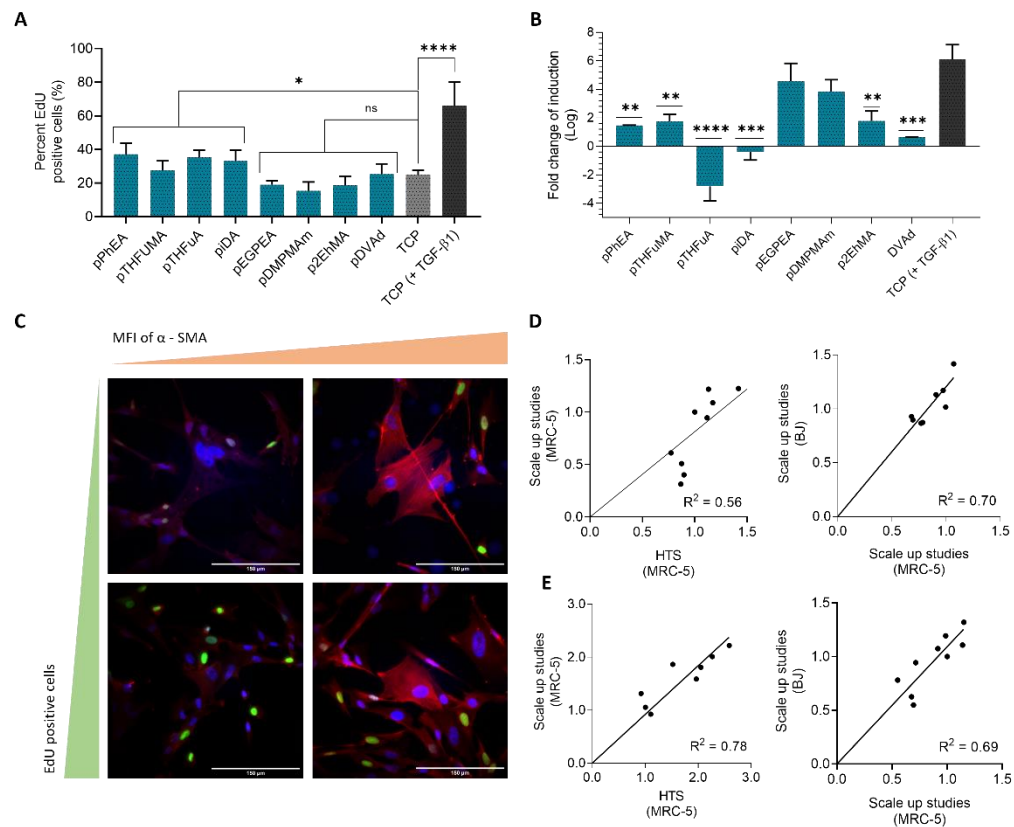


Figure 5.2: Fibroblast proliferation and expression of α -SMA on scaled-up polymers. Fibroblasts were cultured for 96 hours before assessment for A) Cell proliferation. By studying the percentage of EdU positive cells. B) Fold induction of SMA gene expression. C) Representative images of proliferating and differentiating fibroblasts D) Correlation plots for cell proliferation between high throughput studies and scaled up studies, and between lung and skin fibroblasts on scaled up polymer films E) Correlation plots for α -SMA expression between high throughput studies and scaled up studies, and between lung and skin fibroblasts on scaled up polymer films. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

environment for study of fibroblast phenotype. The results indicated the proliferative modulatory ability of the polymers was conserved from the array to the scaled-up phase, as was inferred with an $R^2 = 0.56$, (Figure 5.2D). Moreover, both lung (MRC-5) and skin (BJ) fibroblasts exhibited similar proliferative profiles, having an $R^2 = 0.70$.

Skin (BJ) fibroblast cultured in the pro-fibrotic had a significantly higher percentage of EdU positive cells compared to TCP; this was expected as TGF- β 1 is known to promote fibroblast proliferation. In comparison, the percentage of EdU positive fibroblasts on polymer films ranged from highly proliferating, with pPhEA at 35% EdU positive cells, and with pDMPMAm at 15% EdU positive cells having lowest EdU positive cells, (Figure 5.2A). Based on their modulatory ability, the polymer films could be grouped into i) polymers that enhanced proliferation ii) decreased proliferation and iii) had proliferation comparable to TCP.

Alongside proliferation the fibroblasts were studied for differentiation into α -SMA expressing myofibroblasts. These cell types have been implicated in progressing a scarring and fibrotic outcome. As with proliferation, the α -SMA intensity measurements of the cells cultured on both polymer films and the microarray had a positive correlation of $R^2 = 0.78$, (Figure 5.2E). Furthermore, both MRC-5 and BJ cells had similar α -SMA intensity measurements on the polymer films, with an $R^2 = 0.69$. These positive correlations inferred that the polymers were successfully scaled into larger films, and that they modulated fibroblast differentiation irrespective of tissue source. Moreover, the fold change

in SMA gene expression positively correlated with MFI measurements, having an $R^2 = 0.83$, (Figure A7 in Appendix 4).

Compared to TCP, the gene expression of BJ fibroblasts cultured on all polymer films, with exception of pTHFuA and piDA, was upregulated, (Figure 5.2B). Interestingly, cells cultured on pEGPEA and pDMPMam had highest fold induction with 4.5- and 4.2-fold increase, which was comparable to cells cultured on TCP (+TGF-B1). Whereas fibroblasts cultured on pTHFuA and piDA downregulated SMA gene expression with fold inductions of -2.2 and -0.8, respectively.

5.3.3 Analysis of fibroblast secretome on polymer films

5.3.3.1 Cytokine profile of fibroblasts cultured on polymer films

Fibroblasts were cultured for 96 hours prior to assessment of cell secretome. The concentrations of pro-healing/anti-fibrotic cytokines (bFGF and HGF) and anti-healing/pro-fibrotic (IL-6 and MCP-1) were quantified using a sandwich ELISA. Fibroblasts cultured on polymer films secreted significantly lower concentrations of bFGF compared to TCP and TCP (+TGF-B1) controls, (Figure 5.3A). Further, the polymers could be grouped into two: fibroblasts that expressed **i)** higher mean concentration of bFGF, such as pPhEA, pTHFuMA, pTHFuA and piDA and **ii)** lower mean concentrations, these included pEGPEA, pDMPMam, p2EhMA and pDVAd. Interestingly, this grouping was not observed with HGF secreted by fibroblasts, (Figure 5.3B). The cells cultured on pTHFuMA and pTHFuA films had significantly higher concentrations of HGF, while fibroblasts cultured on pEGPEA, pDMPMam and pDVAd films secreted significantly lower

concentrations of HGF compared to TCP controls. Interestingly, fibroblasts cultured on TCP (+TGF-B1) had comparable HGF secretion to cells cultured on pEGPEA and pDMPMAM films.

The pro-inflammatory cytokine IL-6 was differentially expressed by fibroblasts cultured on the polymers, (Figure 5.3C). In comparison to cells cultured on TCP, the fibroblasts cultured on all polymers with exception of pTHFuMA, pDMPMAM and pDVAd films had significantly lower expression of IL-6. A similar trend was observed with cells cultured on TCP (+TGF-B1); there was a 20-fold decrease in concentration compared to fibroblasts cultured on TCP controls. The monocyte chemoattractant, MCP-1, was significantly highly secreted by fibroblasts cultured on the polymer films; pPhEA, pTHFuMA, piDA, pEGPEA, pDMPMAM and p2EhMA compared to both TCP and TCP (+TGF-B1), (Figure 5.3D). Moreover, the pro-fibrotic cytokine, TGF- β 1, effected fibroblast secretions by decreasing MCP-1 expression as was observed with a decreased concentration between the TCP and TCP (+TGF-B1) controls.

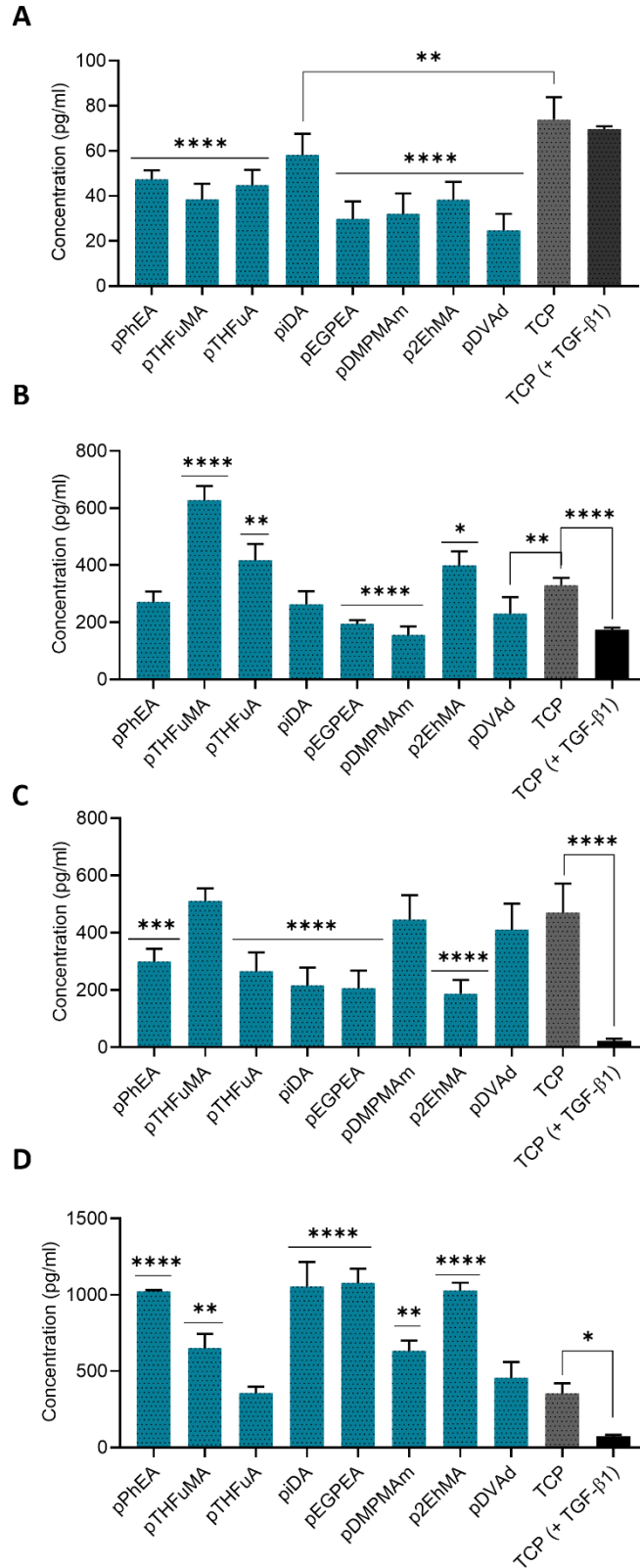


Figure 5.3: Cytokine profile of fibroblasts cultured on scaled-up polymers. The cytokine profile of fibroblasts cultured on polymer films for 96 hours was measured with a sandwich ELISA. A) bFGF B) HGF C) IL-6 and D) MCP-1. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.3.2 Effect of polymer coatings on expression of ECM components

To further study fibroblast secretome, the cells were assessed for expression of collagen I ($\alpha 1$) and collagen III ($\alpha 1$) at mRNA level. All acquired data was normalized to GAPDH controls, and then plotted as fold change to TCP controls. The inclusion of fibroblasts cultured on TCP with exogenously added pro-fibrotic growth factor TGF- $\beta 1$ (TCP (+TGF-B1)), presented a methodology to assess gene expression relative to a fibrotic cellular microenvironment.

Both collagen I and collagen III have been reported as essential ECM components secreted during the proliferative phase of wound healing. Fibroblasts cultured on pro-fibrotic control condition had downregulated collagen III and upregulated collagen I expression, with fold inductions of -0.8 and 1.4, respectively, (Figure 5.4A and B). A similar trend was observed with cells cultured on the polymer films, with exception of pDMPMAm and pTHFuA. The latter upregulated collagen III expression and downregulated collagen I expression.

The collagen III to I ratio has been outlined as an important parameter governing the outcome of wound healing; where higher collagen III to I ratio has been implicated in scarring and fibrosis [42], [44]. Thus, the collagen III to I expression ratio for fibroblasts was plotted, (Figure 5.4C). As was expected the

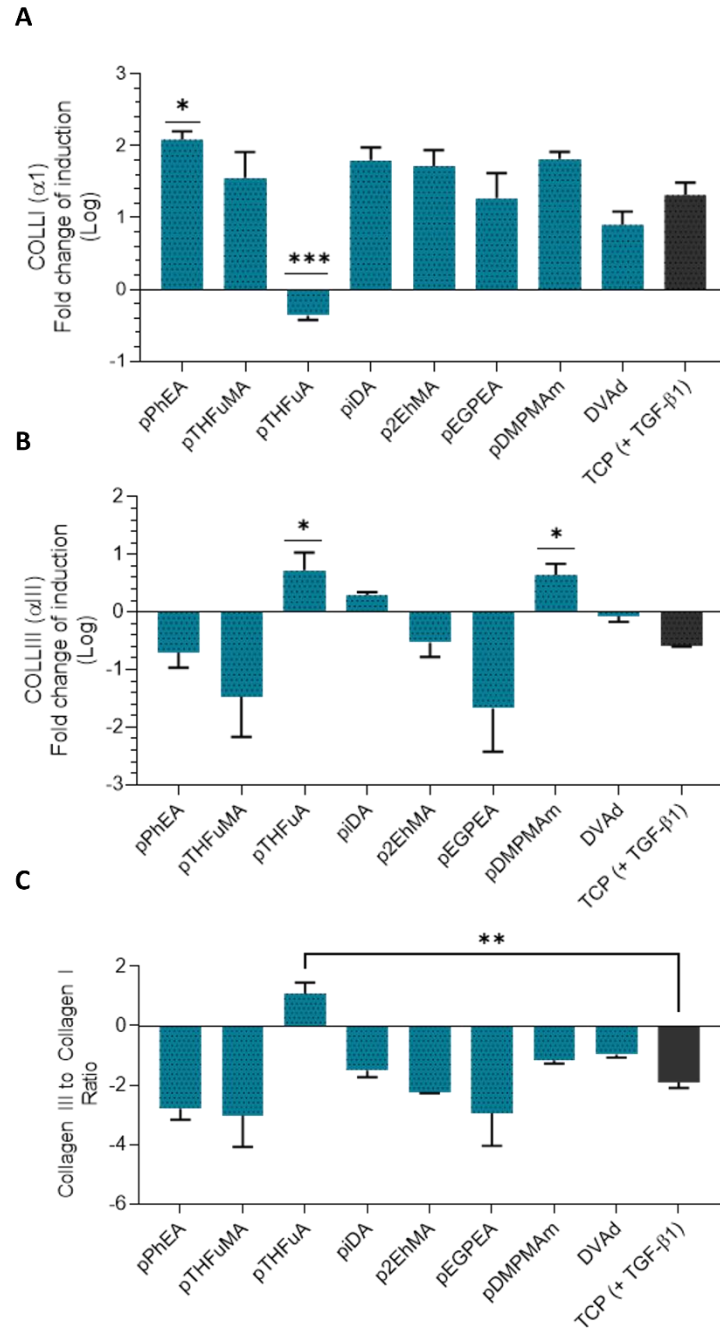


Figure 5.4: **Gene expression for collagens of fibroblasts cultured on scaled-up polymers.** Fibroblasts were cultured for 96 hours prior to studying fold induction in gene expression of A) Coll ($\alpha 1$) I B) Coll ($\alpha 1$) III C) The Coll ($\alpha 1$) III to Coll ($\alpha 1$) I ratio was calculated to observe the differential expression of collagens. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

pro-fibrotic control condition had a positive ratio of -1.8. Furthermore, cells cultured on pPhEA, pTHFuMA, p2EhMA and pEGPEA had higher ratios than

the pro-fibrotic control with ratios below -2. These results suggested that these polymers could modulate the cellular microenvironment towards a pro-fibrotic outcome. Moreover, the collagen ratio on pTHFuA, piDA, pDMPMAM and pDVAd was higher compared to the pro-fibrotic condition. Interestingly, pTHFuA had the highest ratio of 1.3, which was the opposite trend as that observed on the pro-fibrotic control.

5.3.4 Polymer coatings effect wound closure

To assess the impact of polymers on the rate of wound closure, a scratch wound was simulated, into which infiltration of a confluent layer of fibroblasts was studied. Wound closure was assessed by measuring the cell-free area at 48 hours and 96 hours of culture. This methodology enabled accurate assessment of the effect of polymers on proliferative and migratory ability of fibroblasts with regards to wound healing.

The data collected showed that the initial wound area was similar on all polymers and TCP control with an approximate area of $771.6 \times 10^3 \mu\text{m}^2$, (Figure 5.5A). At 48 hours of culture, a differential rate of wound closure was observed. The TCP control had the highest rate of wound closure and the lowest wound area with $319.736 \times 10^3 \mu\text{m}^2$. This was followed by pTHFuA and pDVAd, with a wound area of $393.1 \times 10^3 \mu\text{m}^2$ and $367.9 \times 10^3 \mu\text{m}^2$, respectively, which was significantly lower than the wound area measured on pTHFuMA and pEGPEA surfaces. A similar trend was observed at 96 hours with pTHFuA having significantly lower wound area compared to all other polymers and TCP control. Thus, across the studied time points, pTHFuA had the highest rate of wound

closure followed by the TCP control, the pDVAd, pEGPEA, and pTHFuMA films. This indicated that the fibroblasts cultured on pTHFuA and pDVAd films had greater capacity of wound closure compared to cells cultured on pTHFuMA and pEGPEA films.

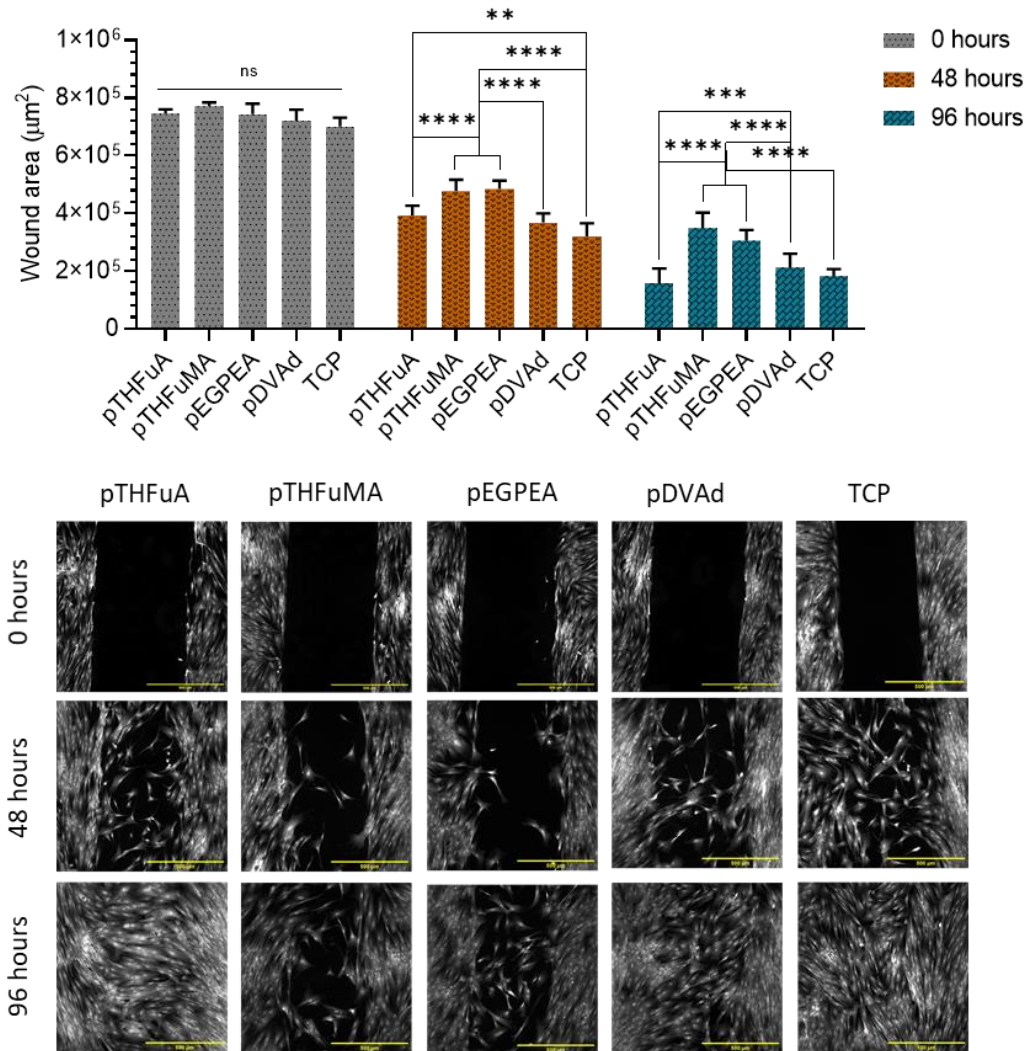


Figure 5.5: Wound healing assay on polymer films. Fibroblasts were cultured on polymer until confluency and wound created. Wound closure was studied by measuring the cell-free area at 48 and 96 hours of culture. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.5 The impact of polymer film stiffness and thickness of bioadsorbate on fibroblast attachment and phenotype

To elucidate mechanism of polymer modulation of fibroblast phenotype, the protein thickness and polymer film stiffness measured in chapter 4 were correlated against fibroblast attachment, proliferation, and differentiation to myofibroblasts. It was observed that fibroblast attachment had a strong positive correlation with thickness of adsorbed proteins on the polymers, (Figure 5.6A). Moreover, protein thickness on polymers had a positive weak correlation with cell proliferation; suggesting that protein thickness was not the strongest variable in modulating cell proliferation. Based on the correlations, fibroblast to

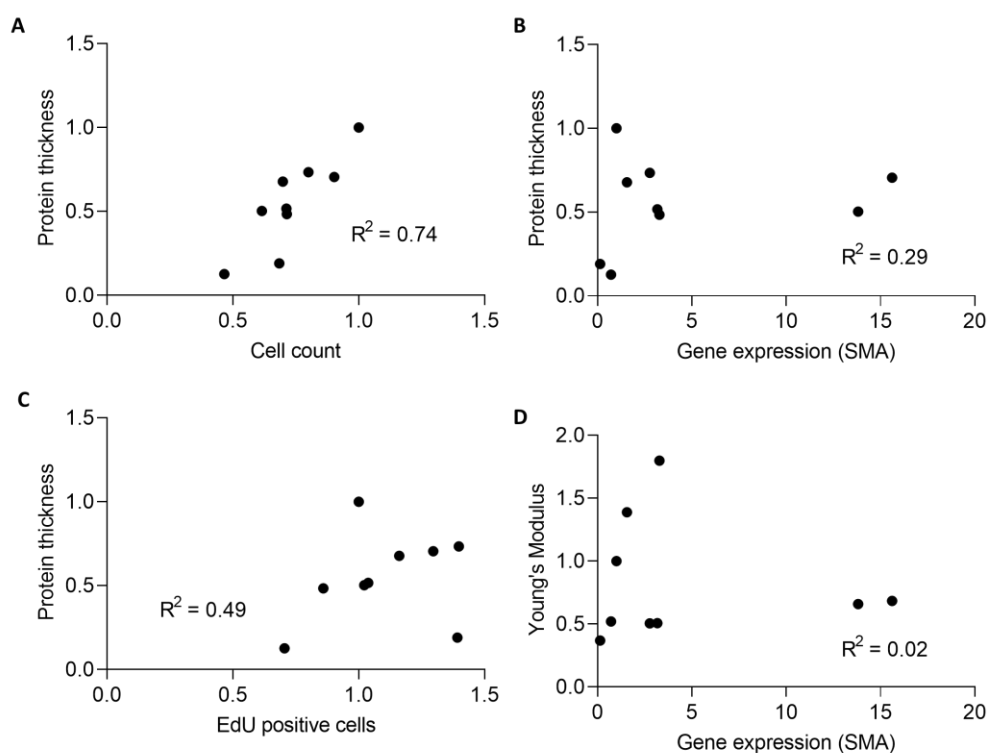


Figure 5.6: Linear regression plots were studied to investigate the mechanisms for polymer modulation of fibroblast behaviour and phenotype. A) Protein thickness measured with XPS and cell attachment B) Protein thickness and fold induction of SMA gene expression C) Protein thickness and cell proliferation D) Stiffness of polymer films and fold induction of SMA gene.

myofibroblast differentiation was not modulated by thickness of adsorbed proteins nor stiffness of the polymer films, (Figure 5.5B and D), having an $R^2 = 0.29$ and $R^2 = 0.02$, respectively. These observations suggested that the polymers modulated fibroblast differentiation via different mechanism, possibly through selective adsorption of proteins from ECM.

5.4 Discussion

Polymers, both natural and synthetic, are increasingly being used in medical applications and devices [126]–[128]. Natural polymers such as alginate and hyaluronic acid (HA) have physiological components that support biocompatibility and have been known to instruct cell behaviour [151]. However, these offer limited control over physiochemical properties, degradation rates and have concerns over sterilization and purification. On the other hand, synthetic polymers offer control over chemical and mechanical properties, degradation rates and can be fabricated into different structures. Specifically, (meth)acrylate and acrylamide polymers have been successfully used as inert filler materials in dentistry, orthopedics, and neuro-prosthetics[129], [157]–[159]. Though their use as cell instructive materials in the context of modulating wound healing and fibrosis is untouched.

Previously, in chapter 4, selected ‘hit’ polymers were synthesised and cast onto coverslips yielding stable polymer films on which fibroblasts were cultured. Initial studies focused on assessing translation of biological efficacy on polymers from the high throughput screen to the scaled-up films. This was evaluated by plotting correlations between high throughput and scaled up data for lung

fibroblast (MRC-5): cell attachment, proliferation, and differentiation. An R-squared value above 0.5 indicated that biological efficacy of polymers was conserved, as was observed for all three studied variables.

Recent studies have highlighted fibroblast heterogeneity in expression and phenotype between tissues [15], [160]. Thus, to address phenotypic modulation of fibroblasts from a heterogeneous tissue source; the phenotype profile of lung fibroblasts (MRC-5) was compared against the phenotype profile of skin fibroblasts (BJ) on the selected scaled up hit polymers. A strong positive correlation ($R^2 > 0.5$) was observed for cell attachment, proliferation, and differentiation into myofibroblasts. This indicated that the polymers modulated both, lung (MRC-5) and skin (BJ), fibroblast behaviour through similar mechanisms. Thus, further phenotypic studies were continued with skin fibroblasts (BJ); the latter representing a cutaneous wound healing model.

Following cytotoxicity studies, the cell adhesion (cell count) of skin fibroblasts (BJ) was studied on polymer films. It was noted that all polymers supported lower cell attachment compared to standard TCP, with exception of pEGPEA which had equivalent cell numbers. Moreover, the polymers had similar measured cell densities with exception of pTHFuA and piDA. Fibroblast adherence is known to be modulated through selective adsorption of serum proteins, specifically fibronectin. To elucidate this further, a correlation plot of adsorbed serum proteins and fibroblast attachment on polymers was plotted. The high positive R^2 value (0.74) indicated that the adsorbed protein thickness influenced cell attachment, where thicker adsorbed protein layer resulted in

higher cell attachment. These findings coincide with other studies that have investigated protein adsorption and cell attachment. Moreover, the adhesion proteins vitronectin and fibronectin have been implicated in promoting cell adhesion. This suggested that though higher adsorbed protein thickness promoted fibroblast attachment on the polymers, the specific mechanism may be selective higher adsorption of vitronectin and fibronectin from serum.

In addition to cell attachment, cell morphology was studied by measuring the aspect ratio and average size of adherent fibroblasts. The analysis of the morphometric data revealed that the cells had two distinct morphologies: spread – polygonal-like and elongated – spindle-like. The morphology (and cell attachment) of cells is known to be modulated by the hydrophilic and hydrophobic properties of the substrates. However, it was observed that fibroblast morphology on these substrates was not influenced by the wettability of the surface, suggesting that other variables such as surface charge and adsorbed fibronectin may have been the dominating factor.

The proliferation of fibroblasts on the polymer films was studied by measuring the percentage of cells that had incorporated EdU in newly synthesised DNA. It was observed that the fibroblast proliferation on the polymer could be grouped into two i) polymers that had mean percentage positive EdU cells greater than TCP and ii) polymers that had mean percentage positive EdU cells equivalent to TCP. Interestingly, it was observed that the polymers that promoted proliferation (in comparison to TCP) had higher molecular weight (>30kDa) as compared to polymers that supported similar proliferative ability to TCP, having molecular

weight (<6kDa). These observations are consistent with previous studies where higher molecular weight polycaprolactone (PCL) films were shown to promote cell proliferation in comparison to low molecular weight PCL films [73]. To further elucidate this mechanism the thickness of adsorbed proteins was correlated against proliferation. The two variables had a weak positive correlation with an $R^2 < 0.5$, suggesting that the thickness of the adsorbed protein layer was not the dominant variable influencing cell proliferation. Studies have elucidated that cell proliferation is modulated not by protein thickness by the amount and conformation of adsorbed fibronectin from serum. Thus, further studies are required to investigate differential adsorption of fibronectin on the polymer films.

The excessive differentiation of fibroblasts to myofibroblasts (that express α -SMA) is the main driver of fibrogenesis and may even occur at reduced levels of inflammation [65]. It was observed that fibroblasts cultured on polymer films had differential fold induction of the SMA gene relative to the TCP control; where cells cultured on pEGPEA and pDMPMAm had similar fold induction to the fibrotic control, TCP (+TGF-B1). Whereas cells cultured on pTHFuA and piDA downregulated SMA gene expression relative to TCP. It has been studied that α -SMA expression is regulated by extracellular stress (polymer stiffness) and by intracellular cell contraction. The latter is controlled by substrate adhesiveness, elastic modulus, and topography to promote cell spreading. To investigate the mechanism via which the polymers modulated SMA expression, correlation plots between protein thickness, polymer stiffness and fold induction

in SMA expression were calculated. Both protein thickness and polymer stiffness had poor correlations with the fold induction in SMA, suggesting that neither thickness of the adsorbed protein nor the stiffness of polymers modulated expression of α -SMA. Interestingly, it was observed that the polymers that downregulated SMA gene expression (relative to TCP) had adsorbed protein thickness less than 1nm. This seems to suggest that selective protein adsorption on the polymers may influence formation of focal adhesions, thereby regulating intracellular contractile elements, expression of α -SMA and subsequent differentiation of cells to myofibroblasts [65]. Furthermore, studies have observed that polymer surfaces, such as pPhEA, are able to drive fibronectin fibrillogenesis thereby promoting cell differentiation[121], [122], [133]. This material driven fibrillogenesis may explain the increased fibroblast differentiation observed on the polymers.

Cytokines and growth factors secreted by fibroblasts influence the cellular microenvironment via autocrine and paracrine signalling; thereby indirectly influencing the outcome of wound healing. The autocrine and paracrine signalling growth factor, bFGF is known to promote fibroblast proliferation, migration and endothelial cell invasion [153], [154]. Moreover, scaffolds designed to release bFGF have been studied to accelerate wound healing by accelerating formation of the granulation tissue[153]. Fibroblasts (BJ) cultured on polymer films had significantly lower expression of bFGF compared to the TCP control. Interestingly, the polymer films that supported higher mean EdU positive cells, these include pPhEA, pTHFuMA, pTHFuA and piDA, also had

higher secretion of bFGF compared to other films. Thus, implicating the growth factor as a possible catalyst via which proliferation is modulated. The anti-fibrotic growth factor, HGF, is studied to inhibit differentiation of fibroblasts into myofibroblasts, promote proliferation and modulate expression of collagens[161], [162]. Fibroblasts culture on TCP (+TGF- β 1) had expressed lower concentrations of HGF than cells cultured on TCP; previous studies have observed similar decrease in expression of HGF when cells are stimulated with pro-fibrotic TGF- β 1[162]. The HGF expressed by fibroblasts on the polymer films varied with pTHFuMA, pTHFuA and p2EhMA having higher concentrations of HGF and pEGPEA, pDMPMAM and pDVAd expressing lower concentrations compared to TCP. These observations suggest that the polymers that promoted expression of HGF could possibly guide the cellular microenvironment towards accelerated wound healing, as was observed with other studies[161]–[163]. The pro-inflammatory cytokine, IL-6, has been studied to upregulate the M-CSF receptor on monocytes directing their differentiation towards macrophages, and to augment fibroblast collagen synthesis[53], [164]; resulting in inflammation and subsequent scar formation. Moreover, studies have observed that a decrease in concentration of IL-6 results in decreased inflammation leading to scarless wound healing[165]. Fibroblasts cultured on pPhEA, pTHFuA, piDA, pEGPEA and p2EhMA secreted lower concentrations of IL-6, compared to TCP. Thus, these polymers may suppress inflammation and direct the cellular microenvironment towards scarless wound healing. The upregulation of fibroblast secreted pro-inflammatory chemoattractant, MCP-1, is observed to instigate migration of macrophages to the wound site,

compounding inflammation. Interestingly, this upregulation is especially crucial in diabetic wounds, where treatment with MCP-1 was studied to enhance wound healing[152]. The cells cultured on pTHFuA and pDVAd had similar expression levels to TCP, suggesting that these polymers inhibited further inflammation. Moreover, polymers such as pPhEA and pEGPEA promoted secretion of MCP-1, thereby possibly furthering inflammation, which may prove beneficial in treating diabetic wounds.

In addition to cytokines and growth factors, the gene expression of collagen I ($\alpha 1$) and collagen III ($\alpha 1$) of fibroblasts cultured on the polymer films was studied. Collagens, specifically collagen I and collagen III, have pivotal roles in skin wound healing. The granulation tissue is mostly composed of collagen III which is progressively replaced by high tensile strength collagen I in the remodelling phase of wound healing. The cells cultured on the polymer films downregulated gene expression of collagen III ($\alpha 1$) relative to TCP, excluding fibroblasts cultured on pTHFuA and pDMPMAm. More so, fibroblasts cultured on all polymers, excluding pTHFuA, upregulated the gene expression of collagen I ($\alpha 1$) relative to TCP. The pro-fibrotic control, TCP (+TGF-B1), downregulated collagen III ($\alpha 1$) expression and upregulated collagen I ($\alpha 1$) gene expression, similar to fibroblast collagen expression on most polymer films. These findings suggested that the collagen expression profile of cells cultured on most polymers followed a fibrotic profile. It has been studied that collagen I and III expression is upregulated on moderately hydrophobic and amine-rich surfaces [42]. This

trend was observed with fibroblasts cultured on pDMPMAm films, where both collagen I and collagen III gene expression were upregulated.

To understand the physiological effect of the collagen secretion profiles, a ratio of collagen III/I was calculated. The collagen III/I ratio has been used to ascertain the outcome of wound healing; for instance, in the case of incisional hernia, the collagen III/I ratio has been known to increase, which resulted in impaired wound healing[42]. Moreover, the low collagen III/I expression ratio of fetal fibroblasts have been studied in conjunction with scarless wound healing[15], [35]. These studies suggest that modulating the collagen III/I expression ratio may guide the outcome of wound healing. It was observed that the pro-fibrotic control, TCP (+TGF-B1), had a low collagen III/I ratio. Fibroblasts cultured on the pPhEA, pTHFuMA, pEGPEA and p2EhMA films had ratios lower than TCP (+TGF-B1) indicating these surfaces promoted scarring, fibrotic healing. Interestingly, fibroblasts cultured on pTHFuA films had a positive collagen III/I ratio which was indicative of scarless wound healing.

The wound healing scratch assay was used to efficiently study the effect of polymer surfaces on wound closure. This methodology demonstrated the direct impact of polymers on fibroblast proliferation and migration, with respect to wound healing. A total of four representative polymers from the eight ‘hit’ scaled-up polymers were studied: these representative polymers supported similar cell numbers and modulated functional fibroblast behaviour such as cell proliferation. The rate of wound closure was assessed by studying the cell-free area after 48 hours and 96 hours of culture. It was noted that fibroblasts cultured

on pTHFuA had the highest rate of wound closure, followed by cells cultured on pDVAd, pEGPEA, and pTHFuMA. These findings suggested that pTHFuA may accelerate wound closure *in vivo*.

The movement of fibroblasts into the wound area as single, non-clustering cells was representative of fibroblast behaviour *in vivo* [166]. Proliferation and migration are the two mechanisms involved in wound closure [155]. Based on observed *in vitro* data, it can be speculated that wound closure on pTHFuA as mediated by both enhanced proliferation and migration of fibroblasts, resulting in efficient wound closure. The pTHFuMA films were observed to promote cell proliferation *in vitro*. However, cells cultured on these surfaces had the lowest wound closure rate suggesting that the polymers suppressed cell migration into the simulated wound. Moreover, the pEGPEA and pDVAd surfaces were observed to suppress cell proliferation. This was notable with decreased rate of wound closure on pEGPEA films. Interestingly, the pDVAd surface had higher rate of wound closure, suggesting that cell migration may be the driving variable. Additional studies investigating proliferation and migration in these wound models would need conducted to further elucidate possible mechanisms.

In summary, hit polymers synthesized, cast, and characterised in chapter 4 were assessed for modulation of fibroblast behaviour towards an anti-fibrotic and pro-fibrotic phenotype. The scaled-up polymer films were shown to retain biological efficacy observed in the high throughput screening phase; this involved studying cell attachment, proliferation, and expression of α -SMA. Moreover, it was noted that the bio-instructive nature of the polymer films was not limited to tissue-

specific fibroblasts: both human lung (MRC-5) and skin (BJ) fibroblasts were shown to behave similarly on the polymers. Further, the polymers were observed to augment the secretion profile of cytokines, growth factors and gene expression of collagens towards distinct pro-healing and pro-fibrotic phenotypes. Based on these results, four polymers that represented each of the classified phenotypic groups (+ CP – SMA, + CP + SMA, - CP – SMA, and – CP + SMA) were selected for the wound healing assay. It was observed that the polymers either accelerated rate of wound closure (pTHFuA) or suppressed the rate of wound closure (pEGPEA). Thus, accentuating the anti- and pro-fibrotic properties of the polymers. In conclusion, these observations emphasize the influential role that polymers may perform in guiding and accelerating wound healing.

Chapter 6: Microparticle aggregates as scaffolds to scaffolds to modulate wound healing

Chapter 6 - Microparticle aggregates as scaffolds to accelerate wound healing

6.1 Introduction

Wound healing is a complex biological process encompassing various cell types across a spatial and temporal matrix[15], [52]. It consists of four overlapping sequential phases, each having defined cell signalling and biochemical events. Following injury, the haemostasis phase results in the formation of a scaffold for infiltrating cells, while acting as a reservoir of growth factors and cells. The inflammatory phase is characterised by infiltration of immune cells, that phagocytize cell debris, microbes, and secrete cytokines to instigate the proliferative phase. The proliferative phase is characterised by proliferation and migration of fibroblasts, myofibroblasts and endothelial cells, which compose the granulation tissue along with the immune cells. Fibroblasts and myofibroblasts are the prevalent cell type in the granulation tissue. These cells secrete structural ECM components that replace the provisional fibrin matrix formed in the haemostasis phase. Fibroblasts remodel this ECM to form mature scar tissue or regenerated skin in the tissue remodelling phase. The balance between these processes determines the outcome of wound healing, in particular fibrosis, scar tissue formation and regeneration of the wound[19], [22], [54].

Accelerated wound healing is an ideal outcome for both acute and chronic wounds. Current strategies to achieve this outcome is to develop i) bioactive dressings such as hydrogel dressings that having hyaluronic acid or chitosan components to promote coagulation and wound healing, ii) microparticles and

nanoparticles as drug delivery agents that release anti-inflammatory and pro-healing drugs and iii) microparticles as cell instructive scaffolds[167]–[170]. The latter has been successfully used in *in vivo* regeneration of cartilage, bone, heart and to accelerate skin wound healing[167].

Microparticles scaffolds can be fabricated with two distinct approaches, by microparticle agglomeration through sintering or the formation of scaffolds mediated by cell aggregation[171]. Both methods produce highly porous scaffolds, permitting cell infiltration while avoiding conventional procedures such as porogen leaching[171], [172]. Microparticle scaffolds mediated by cell-induced aggregation have a distinct advantage over agglomeration; the former avoids invasive surgical procedures for implantation by achieving injectability and scaffold formation *in situ*[172].

Microparticle scaffolds for cell-induced aggregation are commonly functionalized or loaded with cell instructing motifs and growth factors. For instance, chitosan microparticles presenting PDGF-BB, TGF- β 1 and VEGF were shown to improve cell attachment and proliferation of human derived adipose stem cells[173]. In another study, poly(lactic-co-glycolic acid) (PLGA) microparticles were functionalized with cardiac stem cell membrane and secretome that increased adherence and proliferation of cardiac stem cells on the microparticless, while emulating their paracrine and bio-interfacing activities[174], [175]. Alternatively, microparticles fabricated with cell instructive surface chemistries have shown potential in modulating cell

response[134]. This methodology reduces the additional functionalization steps while presenting the cells with a uniform, consistent instructing surface.

In this chapter, the acrylate and acrylamide homopolymers previously identified to modulate fibroblast behaviour were synthesised into surfactants and subsequently fabricated into microparticles. These particles were characterised for surface chemistry, size and stiffness. Furthermore, the particles were then assessed on their efficacy to modulate fibroblast and macrophage behaviour towards anti and pro-fibrotic phenotypes.

6.2 Materials and methods

6.2.1 Microparticle fabrication

Microparticles were fabricated by collaborators at the Centre of Additive Manufacturing, as previously described [134]. In brief, the polymer microparticles were produced using a 100µm hydrophilic 3D flow-focusing microfluidic droplet generator, with the continuous phase as distilled water and the dispersed phase containing the monomer (1,6 hexanediol diacrylate, (HMDA)) with 2% w/v polymer surfactant (THFuA-co-MPEGMA, and EGPEA-co-MPEGMA) and 1% w/v photoinitiator (2,2 dimethoxy-2-phenylacetophenone). The emulsions were then collected in distilled water and irradiated with UV light at 365nm. The microparticles were then characterised for size and surface chemistry.

6.2.2 Cell culture and seeding

The human skin fibroblasts (CRL-2522, ATCC) were cultured in Minimum Essential Medium Eagles (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, non-essential amino acids, penicillin/streptomycin and

sodium pyruvate (Sigma). The cells were cultured in T75 flasks at 37°C with 5% supplemental CO₂ until 90% confluent before passaging. Fibroblasts were seeded with a density of 2500 cells/mg microparticles in a non-TCP U-bottom plate (Corning).

6.2.3 Monocyte culture

Buffy coats were obtained from healthy donors (National Blood Service, Sheffield, UK) after informed written consent and following ethics committee approval (Research Ethics Committee, Faculty of Medicine and Health Sciences, University of Nottingham). Monocytes isolated from buffy coats were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and penicillin/streptomycin (Sigma). The cells seeded on microparticles and on M0 polarized controls were supplemented with macrophage colony stimulating factor (M-CSF, 10 ng/ml). The M1 and M2 polarized controls were supplemented with IFN- γ (20 ng/ml) + GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) + M-CSF (50 ng/ml) respectively. The cells were cultured at 37°C with 5% CO₂ for a total of 6 days. Fresh medium containing cytokine polarization cocktails was supplemented at day 3 of culture. The cultured cell supernatant was then collected and stored at -80°C until analysis.

6.2.4 Cytotoxicity

The cell viability of fibroblasts on the microparticles was assessed using the ToxiLight™ non-destructive cytotoxicity bioassay kit (Lonza) at 24 hours of culture. The assay measures the release of adenylate kinase (AK) from cells with compromised cell membranes. The AK catalyses conversion of ADP to ATP,

which is then detected through bioluminescence. The emitted light intensity is linearly related to the AK concentration, and can be detected using a plate reader.

6.2.5 Cell attachment

Cell attachment of fibroblasts was measured using the CyQuant™ NF assay (ThermoFisher) at 24 hours of culture. This method is based on measurement of cellular DNA content via fluorescent dye binding, where the cellular DNA content is proportional to the number of cells. The DNA content of fibroblasts cultured on microparticles was measured and their fluorescence intensity was compared against fluorescence intensity of known cell seeding densities.

6.2.6 Cell proliferation

Cell proliferation was measured with the Baseclick EdU flow cytometry kit (Baseclick). EdU was added to cell medium at 3μM at 72 hours of culture, where it was incorporated into newly synthesised DNA during the G1/S phase of the cell cycle. After 96 hours, cells were detached, and the cell-microparticle suspension was sieved with a 50μm sieve to collect cells. The cells were stained for EdU following manufacturer's instructions. The data was acquired on BD FACS Canto A and analysed using Kaluza.

6.2.7 ELISA

The concentrations of bFGF, HGF, IL-6, MCP-1, and TGF-β1 secreted into the medium by fibroblasts cultured on microparticles was analysed after 96 hours of culture with DuoSet ELISA kits (R&D Systems) according to manufacturer's instructions. Similarly, the concentrations of IL-10, CCL18, TNF-α, IL-1β, and

TGF- β 1 secreted into medium by macrophages cultured on microparticles were measured after 6 days of culture.

6.2.8 RT-PCR

Total RNA was isolated from cells using RNeasy plus kit (Qiagen) followed by cDNA synthesis using a qPCRBIO cDNA synthesis kit (PCR Biosystems). Real-time PCR was performed on the MxPro 3005P qRT-PCR system (Stratagene, USA) using 2x qPCRBIO SyGreen Blue mix Lo-Rox (PCR Biosystems), according to manufacturer's instructions. Forward and reverse primers were purchased from Eurofins Scientific UK and Sigma, these are listed in table 6.1. Gene expression levels were first normalised to housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then the relative expression levels were calculated with the $2^{-\Delta\text{ct}}$ method.

Table 6.1: Primers for fibroblast ECM markers used in real-time qPCR

Genes	Primer	Sequence (5'-3')
GAPDH	Forward	ACAGTCCATGCCATCACTGCC
	Reverse	GCCTGCTTCACCACCTTCTTG
EDA-Fn	Forward	CCAGTCCACAGCTATTCCTG
	Reverse	ACAACCACGGATGAGCTG
Collagen I (α 1)	Forward	GTCGCACTGGTGATGCTG
	Reverse	GGTGGTGTCCACCTCGAG
Collagen III (α 1)	Forward	AGCTGGAAAGAGTGGTGACAG
	Reverse	CCTTGAGGACCAGGAGCAC

6.2.9 Imaging

CellMask Green (Invitrogen) was used to visualize cells adherent on microparticles. The samples were imaged using the Zeiss LSM710 confocal with a Zeiss Observer microscope (Carl Zeiss) under a 40x oil objective.

6.2.10 Microparticle surface characterisation

The microparticle characterisation was carried out by collaborators at the Centre of Additive Manufacturing, and at the School of Pharmacy.

Microparticle surface chemistry was measured using ToF-SIMS IV (IONTOF GmbH, Germany) equipped with a Bi^{3+} primary ion source. Data was calibrated and analysed using IonToF software.

The MFP-3D Standalone Atomic Force Microscope (AFM) (Oxford Instruments, Asylum Research Inc., CA) was used to obtain force-displacement curves of the polymer samples in water for Young's modulus calculation. Following data collection, the Derjaguin-Muller-Toporov mathematical model was used to fit the slope of the retracting curve using least squares regression line for calculation of Young's modulus.

6.2.11 Statistical analysis

All data acquisitioned is expressed as mean $\pm$ standard deviation, with two independent biological replicates, and three technical replicates each. Statistical significance was calculated using one-way ANOVA and the Tukey's post hoc analysis, whereby $p \leq 0.05$ was considered as being statistically significant.

6.3 Results

6.3.1 Microparticle surface characterisation

The microparticles generated by droplet microfluidics produced monodisperse particles with particle diameter of approximately $70\mu\text{m}$, (Figure 6.1C and E). The size distribution for all particles was narrow, ranging between $2.29\mu\text{m}$ and $3.32\mu\text{m}$. Furthermore, the pro-fibrotic particles had higher mean surface modulus than anti-fibrotic particles with 14.8GPa and 9.7GPa respectively, figure 6.1B, though this increment was not statistically significant. This indicated that the microparticles had similar physical and mechanical properties.

ToF-SIMS analysis was used to investigate the surface chemistry of the produced microparticles. The spectra were collected both in the positive and negative ion mode, to identify characteristic peaks for THFuA ($\text{C}_6\text{H}_5\text{O}^-$), EGPEA ($\text{C}_5\text{H}_9\text{O}^+$), and HMDA, where no characteristic peak could be identified, (Figure 6.1A). Comparison of ion peaks of THFuA and EGPEA particles with HMDA core particles show that the ions were unique to the individual particles suggesting that the particles were functionalized with the biologically active polymers.

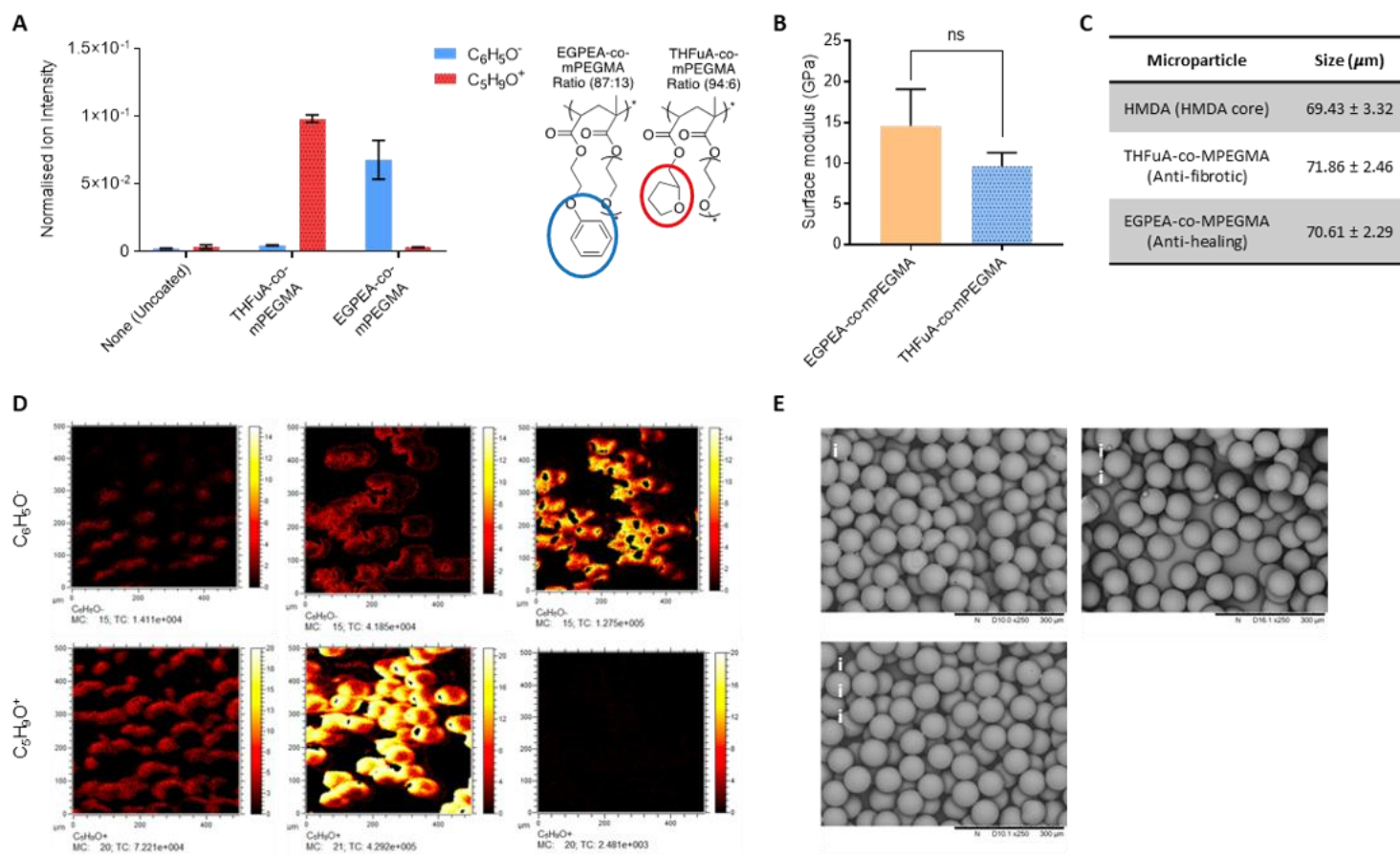


Figure 6.1: Surface characterisation of fabricated microparticle. A) ToF-SIMS data showing 3 key ions associated with 3 monomers with the surfactant structures, with the ions from the structures circled in blue and red circles. B) Stiffness of microparticles, both particles had similar surface modulus. C) Table with size of microparticles D) SEM of monodisperse microparticles

6.3.2 Cytotoxicity and cell attachment

The microparticles were washed for 48 hours in distilled water prior to UV sterilization and cell culture. The water wash was implemented to leach and remove any residual toxicants. Fibroblasts seeded onto the microparticles were assessed for toxicity by measuring the release of AK from lysed cells. All particles had high cell viability comparable to the live control, (Figure 6.2A).

The CyQuant assay was used to assess the total DNA on the microparticles after 24 hours of culture. The anti-fibrotic particles had significantly higher cell attachment with 32×10^3 cells, compared to pro-fibrotic with 22×10^3 cells, (Figure 6.2B). The HMDA core particles supported higher mean attachment than pro-fibrotic, though this was not statistically significant. Moreover, the non-TCP

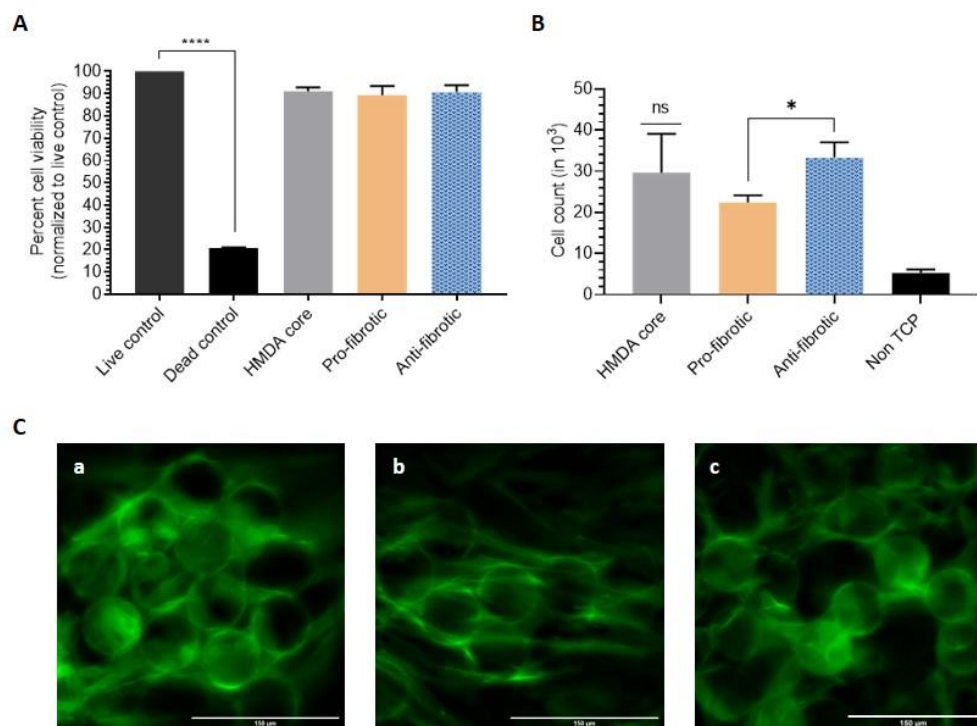


Figure 6.2: Fibroblast cytotoxicity and attachment on microparticles. A) Cytotoxicity B) Cell attachment C) Representative images of fibroblasts stained with CellMask Green a) HMDA core b) pro-fibrotic c) anti-fibrotic. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

well had a fifth of the cells attached compared to the anti-fibrotic particles. This suggested that cells were adherent on all three microparticles, and that attachment was guided by particle chemistry alone.

6.3.3 Fibroblast proliferation on microparticles

Fibroblast proliferation on the microparticles was studied after 96 hours of culture. At 72 hours, medium was replaced with EdU containing medium. The percentage of EdU positive cells was then measured, this indicated the percentage of cells that were in the G1/S phase of their cell cycle. A shift in peaks between the stained and unstained controls confirmed incorporation of EdU into

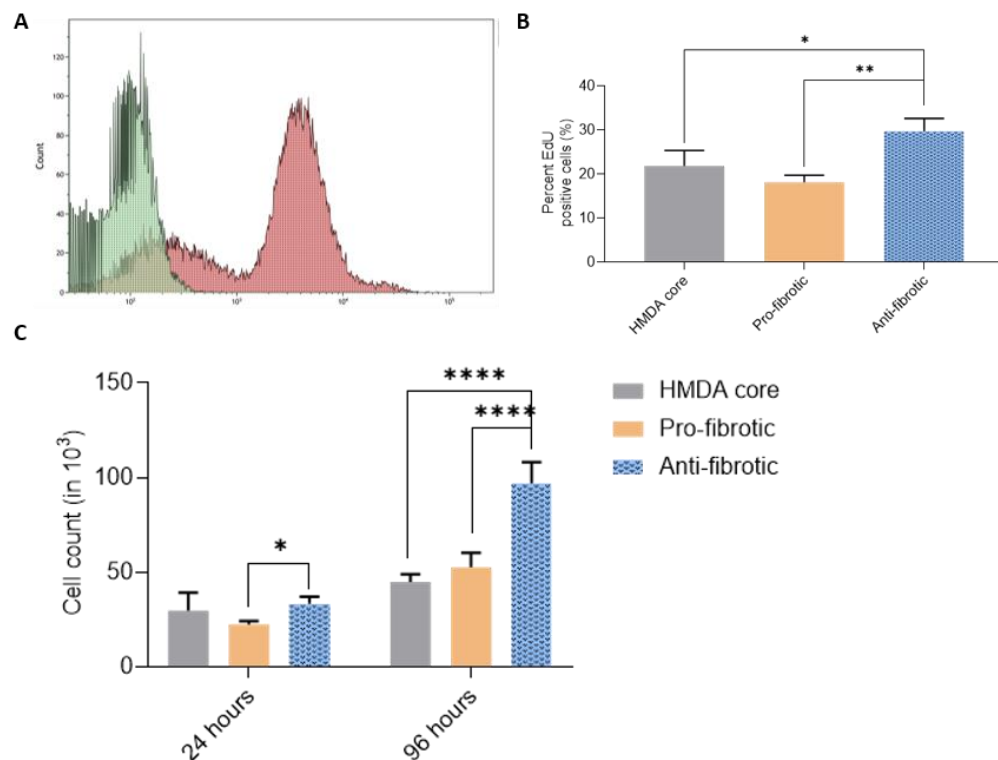


Figure 6.3: Cell proliferation of fibroblasts on microparticles. A) Fibroblasts negatively for EdU are to the left (green) and positively stained for EdU on the right (red). B) The percentage positive EdU cells were measured from the flow scatter plots. The pro-healing particles had significantly higher cells in their G1/S phase of the cell cycle. C) Additionally, cell proliferation was studied by quantifying the total number of cells at 24 hours and 96 hours of culture. The pro-healing particles had significantly higher population of cells at both 24 hours and 96 hours of culture. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

proliferating cells, figure 6.3A. The anti-fibrotic microparticles had significantly higher percentage of EdU positive cells compared to both pro-fibrotic and HMDA core particles, with 29%, 18% and 21% EdU positive cells respectively, (Figure 6.3B). A similar trend was observed with a significantly higher population of adherent cells on anti-fibrotic particles at 96 hours (Figure 6.3C); suggesting that the anti-fibrotic microparticles promoted cell proliferation compared to pro-fibrotic and HMDA core microparticles.

6.3.4 Microparticles influence fibroblast cytokine profile

Fibroblast supernatant cultured on microparticles was collected after 96 hours and cytokine concentrations were measured with a sandwich ELISA assay. A panel of 5 cytokines were assessed: bFGF, HGF, IL-6, TGF- β 1, and MCP-1, (Figure 6.4). Fibroblasts cultured on anti-fibrotic microparticles secreted significantly higher concentrations of bFGF and HGF than the pro-fibrotic

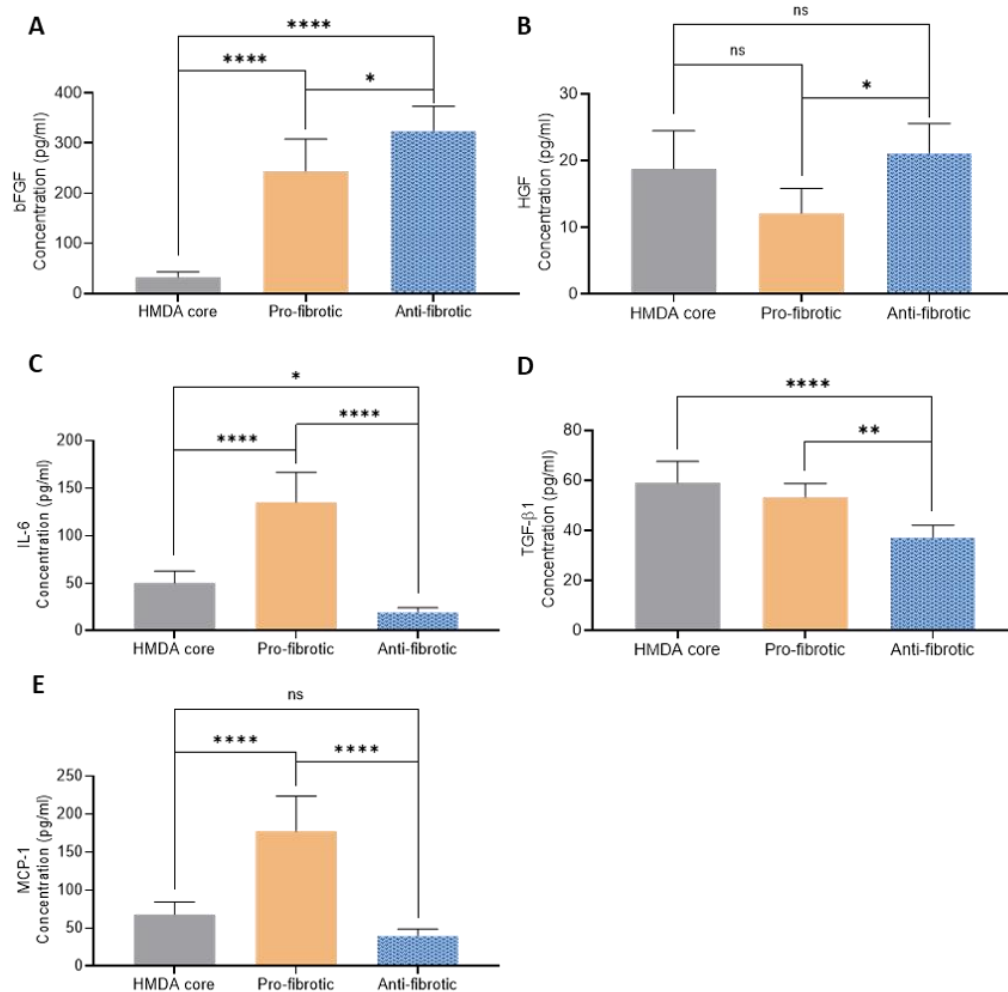


Figure 6.4: Cytokine profile of fibroblasts cultured on microparticles. Human skin fibroblasts were cultured on microparticles for 96 hours after which the culture supernatant was analysed for concentrations of A) bFGF B) HGF C) IL-6 D) TGF- β 1 and E) MCP-1. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

microparticles. The pro-fibrotic cytokines, IL-6 and TGF- β 1, concentrations were significantly higher with fibroblasts cultured on pro-fibrotic particles than anti-fibrotic particles. A similar trend was observed for MCP-1, where fibroblasts cultured on pro-fibrotic particles secreted higher concentrations than anti-fibrotic particles. These trends suggested that fibroblasts cultured on the functionalized particles adopted a anti-fibrotic or an pro-fibrotic secretion profile. Interestingly, cells cultured on the HMDA core microparticles did not have strict anti- or pro-fibrotic secretion profiles.

6.3.5 Gene expression of fibroblasts on microparticles

The mRNA levels of certain ECM markers of fibroblasts cultured on microparticles were studied using real time - qPCR. The fold induction in gene expression was measured compared to the HMDA core particles. Fibroblasts cultured on anti-fibrotic particles expressed lower levels of all three ECM markers studied, in comparison to pro-fibrotic particles, (Figure 6.5). ED-A fibronectin is a variant of fibronectin secreted by myofibroblasts, the latter differentiating from fibroblasts. The two-fold increase in ED-A fibronectin mRNA levels between pro- and pro-fibrotic particles suggest that the pro-fibrotic particles had a higher population of myofibroblasts. Similarly, the pro-fibrotic particles had approximately two-fold increase in collagen III expression than anti-fibrotic microparticles. Interestingly, the anti-fibrotic particles downregulated while pro-fibrotic particles upregulated collagen I expression. relative to HMDA core particles, suggesting an instructive nature of the particles.

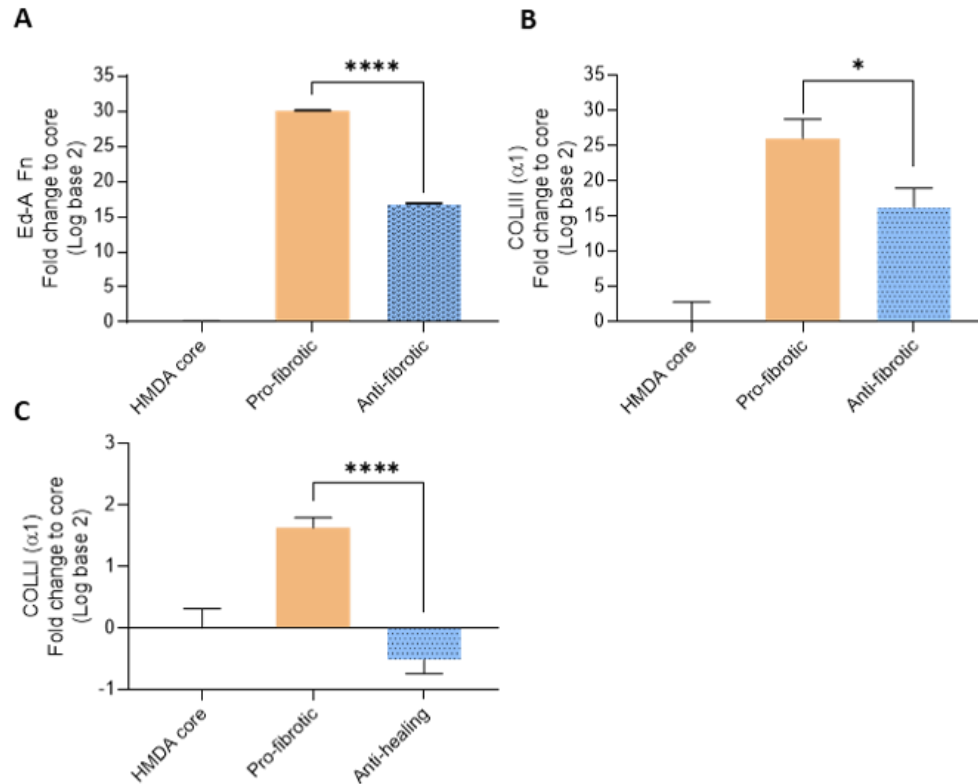


Figure 6.5: **Gene expression of ECM markers for fibroblasts cultured on microparticles.** Relative fold induction in gene expression of A) EDA- FN B) Collagen III ($\alpha 1$) C) Collagen I ($\alpha 1$) using GAPDH as housekeeping gene. The fold induction was calculated using the $2^{-\Delta Ct}$ method and data was normalized to HMDA core particles. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.3.6 Cytokine profile of macrophages on microparticles

Macrophages were cultured on microparticles for 6 days before collecting cell culture supernatant and studying their secretome. The studied cytokines were grouped into anti-inflammatory, IL-10 and CCL18, pro-inflammatory, TNF- α and IL-1 β , and pro-fibrotic factor TGF- $\beta 1$, (Figure 6.6). The polarized macrophage controls for both anti-inflammatory cytokines, IL-10 and CCL18, were significantly higher in M2-like macrophages, as was expected. Macrophages cultured on anti-fibrotic microparticles had significantly higher expression of IL-10 compared to pro-fibrotic particles. Moreover, the cells had

higher mean secretion of CCL18 on anti-fibrotic particles than pro-fibrotic particles. This suggested that cells cultured on anti-fibrotic particles adopted a M2-like secretome profile. On the other hand, the pro-fibrotic particles influenced macrophages to secrete higher concentrations of pro-inflammatory TNF- α than the anti-fibrotic particles. Interestingly, macrophages cultured on pro-fibrotic particles secreted significantly higher concentrations of pro-fibrotic growth factor TGF- β 1. This indicated a pro-inflammatory and pro-fibrotic cytokine profile for macrophages cultured on pro-fibrotic particles.

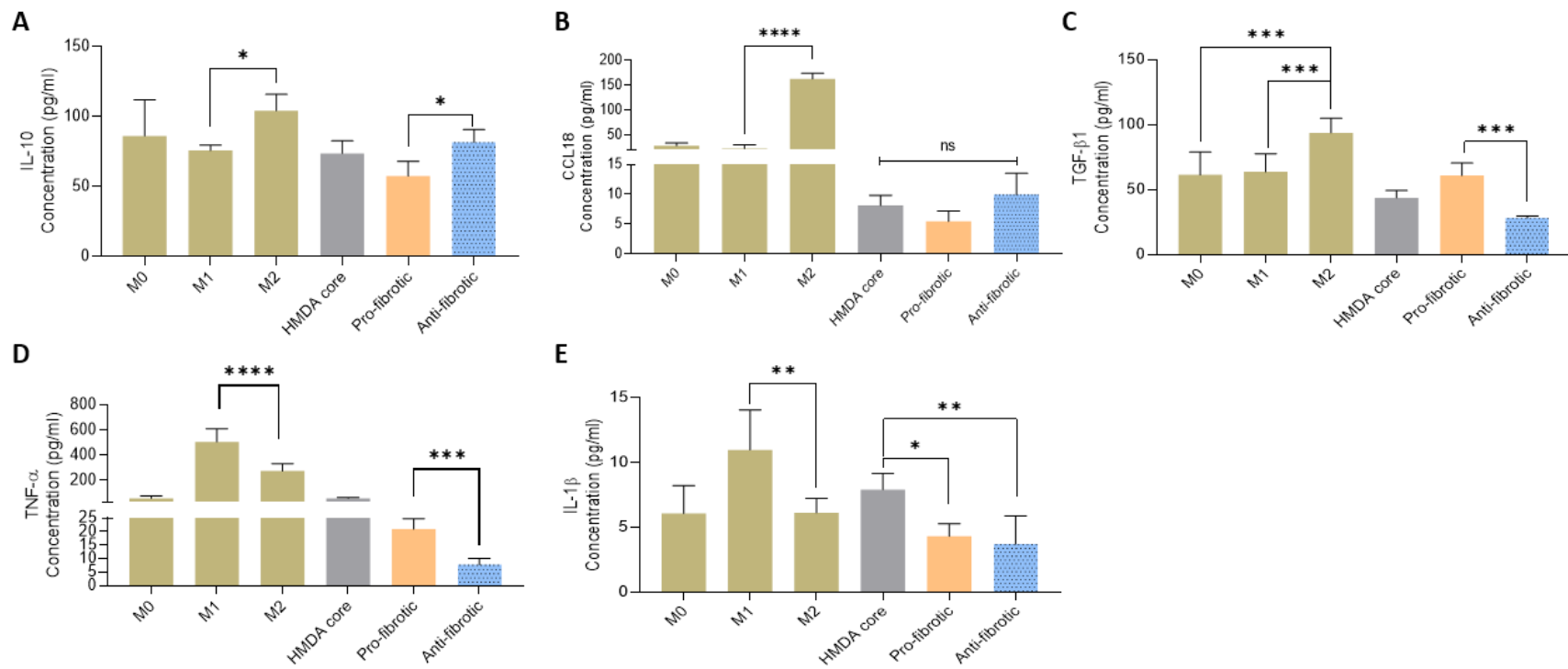


Figure 6.6: **Cytokine profile of macrophages cultured on microparticles.** Monocytes were differentiated into macrophages on the polymers for 6 days prior to collecting culture supernatant and studying the concentrations of A) IL-10 B) CCL18 C) TGF-β1 D) TNF-α and E) IL-1β. All data shown is from two biological replicates with three technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.4 Discussion

Microparticle scaffolds have been successfully utilized as a bottom-up engineering approach of complex 3D constructs for use as injectables for *in vivo* regeneration of cartilage[169], [176], [177], bone[168], [178], and heart tissues[174], [175]. However, their use as instructive scaffolds for modulating and accelerating wound healing is limited[64], [167]. The discovery of polymers that modulated fibroblast behaviour towards an anti-fibrotic or pro-fibrotic phenotype presented an opportunity to study polymeric microparticle scaffolds with cell instructive chemistries that could regulate the outcome of wound healing processes.

In this study, acrylate polymers identified from the high throughput screening and scaled up studies were translated into polymer surfactants. These surfactants were then fabricated into microparticles using a microfluidics approach. The produced microparticles had a monodisperse size distribution with the particles being 70 μ m in diameter, as measured by the SEM images. Further characterisation revealed that the particles had defined surface chemistry, with anti-fibrotic (pTHFuA) or pro-fibrotic (pEGPEA) chemistries, and with similar mean surface modulus at 10GPa and 15GPa respectively. Thus, the fabricated microparticles were similar in all aspects, with surface chemistry as the delineating factor.

Primarily biomaterial surfaces should be able to support cell attachment and proliferation, which could then regulate other downstream processes such as cytokine and protein expression. Fibroblast attachment and proliferation on

microparticles was measured by correlating the total DNA extracted from the particles to a cell number, using a calibration curve. Additionally, cell proliferation was studied by assessing the percentage of EdU positive cells and the total number of cells at 24 and 96 hours of culture.

All microparticles supported cell attachment, there-in the anti-fibrotic particle supported highest cell attachment followed by HMDA core and pro-fibrotic microparticles. It is known that cell attachment is regulated by adsorption of ECM proteins, namely fibronectin, from serum. Furthermore, fibronectin has been used to enhance cell attachment to microparticles *in vitro* [174], [179]. This suggests that the differential attachment of fibroblasts on the microparticles may be related to varying adsorption levels of fibronectin. Interestingly, in a study conducted by Ugur et al. (2020), it was noted that microparticle properties such as zeta potential and particle morphology were not solely responsible for high attachment of mesenchymal stem cells[179]. The study elucidated that concentrated patchy adsorption of fibronectin may result in enhanced cell attachment and proliferation. Thus, it can be hypothesized that the differential attachment of fibroblasts on the microparticles is associated with varying levels of concentrated patches of fibronectin.

In addition to serving as an *in-situ* scaffold for attachment and proliferation the microparticles influenced fibroblast and macrophage secretome. A panel of cytokines comprising both pro-fibrotic and anti-fibrotic cytokines were assessed. Cells cultured on anti-fibrotic microparticles had significantly higher concentrations of bFGF, HGF and IL-10 in comparison to the pro-fibrotic

particles. On the other hand, cells cultured on pro-fibrotic particles had significantly higher concentrations of pro-fibrotic TGF- β 1, IL-6, MCP-1, TNF- α and IL-1 β . These secretion profiles aligned with anti-fibrotic and pro-fibrotic phenotypes, suggesting that the microparticles influenced cell secretions towards the intended outcomes.

Secreted cytokines in the cellular microenvironment influences cell behaviour and phenotype, such as proliferation and ECM secretion, which ultimately influence the outcome of wound healing. For instance, the autocrine effect of fibroblast secreted bFGF enhances proliferation. This is observed on fibroblasts cultured on anti-fibrotic microparticles, where high concentrations of bFGF coincide with higher proliferative index. Interestingly, the anti-fibrotic growth factor HGF is known to accelerate wound healing and prevent fibrosis; by synergistically acting with bFGF to promote proliferation and modulating the effects of pro-fibrotic cytokine TGF- β 1 [161]. It was observed that fibroblasts culture on anti-fibrotic microparticles secreted higher concentrations of HGF, which may allude to the anti-fibrotic effects of the particle.

Fibroblasts and macrophages have been identified as the two major cell types dictating the outcome of wound healing. The impact of fibroblast-mediated recruitment of macrophages through paracrine signalling was investigated by studying concentrations of MCP-1 and IL-6. The former acts as a chemoattractant for monocytes while the latter upregulates the M-CSF receptor on monocytes furthering their differentiation into macrophages[180]. Fibroblasts cultured on pro-fibrotic particles expressed higher concentrations of both MCP-

1 and IL-6, which may instigate influx of macrophages into the microparticle scaffold system thereby furthering inflammation. Moreover, macrophages cultured on the microparticles expressed distinct M1-like or M2-like cytokine profiles, as was observed in comparison to the cytokine cocktail polarized controls. Macrophages cultured on anti-fibrotic microparticles secreted higher concentrations of IL-10, which has been observed to dedifferentiate myofibroblasts into fibroblasts, thereby providing an anti-fibrotic effect[55]. Furthermore, macrophages cultured on pro-fibrotic particles secreted higher concentrations of pro-inflammatory TNF- α and pro-fibrotic TGF- β 1, which may then promote a pro-fibrotic microenvironment.

Collagen is the main ECM protein and is essential for providing mechanical strength to regenerated tissues. In healthy tissues, collagen I is the prevalent compared to collagen III, although healing tissues are known to have higher collagen III content which is replaced with collagen I in later stages of wound healing. Moreover, an abundance of collagen III has been noted in acutely or chronically inflamed tissues[44]. It was observed that fibroblasts cultured on anti-fibrotic particles expressed a lower collagen III to I ratio than fibroblasts cultured on pro-fibrotic particles. The expression of higher collagen III to I ratio in the early stages of wound healing has been studied to be an early indicator of normal healing[54], [148]. On the contrary, a lower collagen III to I ratio was observed in mature burn scar tissue. These findings suggest that the anti-fibrotic particles promoted a normal expression of collagen III to I, whereas the pro-fibrotic particles may lead towards excessive ECM deposition and scarring. The

upregulation of collagens on fibroblasts cultured on pro-fibrotic particles, can be attributed to the increased secretion of TGF- β 1 and IL-6, which is a key enhancer of collagen production [66]. Moreover, the anti-fibrotic growth factor HGF attenuates expression of collagens [161], this was observed on anti-fibrotic particles where the collagen I and III expression levels were significantly downregulated compared to pro-fibrotic particles..

Along with collagen expression, the expression of cellular derived ECM protein ED-A fibronectin was studied. This variant of fibronectin is expressed by fibroblasts and myofibroblasts during the proliferative phase of wound healing and has been observed to be abundant in fibrotic tissue[181], [182]. It has been studied that ED-A fibronectin regulates binding of latent TGF- β 1, thereby furthering the fibrotic outcome. The pro-fibrotic microparticles had higher expression of ED-A fibronectin, which may be a result of higher mean surface modulus of these particles, promoting fibroblast differentiation to myofibroblasts and subsequent ED-A fibronectin expression. Furthermore, the increased concentration of fibroblast secreted TGF- β 1 on pro-fibrotic particles may be elucidated by the increased availability of binding sites for latent TGF- β 1 on expressed ED-A fibronectin [183].

Fibroblast phenotype is influenced by material chemistry, stiffness, and topography. As mentioned earlier, the surface characterisation of the microparticles revealed that the fabricated particles had similar surface modulus and a smooth topography. The differences in fibroblast attachment between pro- and pro-fibrotic particles could then be attributed to microparticle surface

chemistry alone. The mechanism with which microparticle chemistry influences cell behaviour requires further investigation, though mechanistic parallels with flat films may be drawn.

The *in-situ* scaffold formation with microparticles added an additional dimension to the 2D studies from chapter 4 and 5. The created scaffold not only introduced a 3D system, but also introduced surface curvature along with chemistry as a variable that influenced cell behaviour. It is known that fibroblasts can differentiate curvature up to 2mm after which the responses are similar to flat surfaces[184], [185]. Moreover, it has been studied that surface curvature has effects on fibroblast spreading, where smaller diameter spheres result in smaller cell spread area [184]. Furthermore, it was observed that curvature could influence nuclear morphology, which in turn may influence gene-expression profiles[186]. Based on these studies it can be construed that *in-situ* fabrication of the porous curved scaffold-like microenvironment may guide actin reorganization and nuclear morphology, resulting in varying cellular processes such as proliferation and differentiation.

To summarise, polymers that modulated fibroblast behaviour towards anti-fibrotic and pro-fibrotic phenotypes were synthesised into surfactants and fabricated into microparticles. The particles had unique surface chemistry with similar size distribution and mechanical characteristics. These microparticles formed *in situ* scaffolds in culture, and modulated fibroblast and macrophage phenotypes and secretome towards anti-fibrotic or pro-fibrotic phenotypes. To better evaluate the effect of the scaffold on the cellular microenvironment a co-

culture system between fibroblasts and macrophages on the microparticle scaffold remains to be investigated. Furthermore, the particles were fabricated with a non-biodegradable core which limits *in vivo* applicability, though it provides a strong proof-of-concept. Further investigations must include fabrication of these particles with a biodegradable core. In conclusion, current results suggest that the microparticle system could potentially be utilized to form *in situ* scaffolds to accelerate wound healing in acute and chronic wounds and to possibly reduce excessive scarring in burn injuries.

Chapter 7: General Discussion

Chapter 7 - General discussion

Wound healing is an intricate biological process involving several overlapping phases that consist of inflammation, formation of granulation tissue, re-epithelialisation, matrix formation and remodelling [15], [49], [52]. These phases involve various cell types such as immune cells, endothelial cells, and stromal cells; each having a defined role that guides wound healing from injury to regeneration. Dysregulation in wound healing phases, such as in the case of diabetic wounds or burn injuries, leads to negative outcomes including chronic non-healing wounds or the formation of excessive scar tissue.

The formation of enhanced granulation tissue has been studied to promote and accelerate wound healing[58], [170]. Fibroblasts and myofibroblasts are the prevalent cell type in the granulation tissue and have been reported to influence the outcome of wound healing [15], [19]. These cells secrete structural ECM components that replace the provisional fibrin and remodel the ECM to form regenerated or mature scar tissue. The balance between these processes determines the outcome of wound healing: fibrosis, scar tissue formation or regeneration of the wound.

Accelerated wound healing is an ideal outcome for both acute and chronic wounds. Current methods to achieve this encompass the development of bioactive dressings/scaffolds and the delivery of growth factors and other agents via micro- and nanoparticles to promote healing [167]–[170]. For instance, the current standard to manage chronic wounds and third-degree burns is the autograft of skin or cultured epithelial cells or the use of bioengineered skin

substitutes. Though these techniques have achieved a degree of success they are observed to cause adverse reactions (rejection, infections, and allergies). Additionally, they have a limited capacity to instigate the healing process without stimulating further remodelling. Hence, the aim of this study was to discover new polymeric biomaterials that could augment and accelerate wound healing. This involved the identification of synthetic polymers that supported fibroblast attachment and modulated their phenotypic profile.

High throughput screening of chemically diverse meth(acrylate) and acrylamide library has previously been successfully implemented to discover simple polymers that could instruct macrophage phenotype or direct pluripotent stem cell expansion and differentiation. Thus, it was hypothesized that this chemically diverse library could be used to identify polymers that modulated fibroblast behaviour towards anti-fibrotic and pro-fibrotic phenotype, thereby augmenting wound healing. The hypothesis was assessed by studying the ability of 300 chemically diverse homopolymers to modulate fibroblast attachment, size, proliferation, and differentiation to α -SMA expressing myofibroblasts. The screening resulted in the discovery of fibroblast-instructive polymers that could guide wound healing. The discovery of these bio-instructive polymers was enabled by rapid assessment of a large polymer library through use of a high throughput screening approach, which otherwise may not have been theorised.

The high throughput screening methodology has several advantages, the most distinct being simultaneous assessment of a large library of surfaces or compounds. However, in this study the methodology limited the range of

phenotypic and functional assays that could be studied. The other limitation in experimental design is the proximity of polymers on the microarray screening platform; resulting in crosstalk (through chemical signalling) between cells cultured on the polymers. This was overcome by designing experimental plans such that hit polymer selection was based on studying multiple technical and biological replicates.

The screening produced a large information rich data set that represented phenotypic and behavioural characterisation of fibroblasts on a diverse array of polymers. This dataset could be analysed using machine learning algorithms to extract information on the relationship between polymer physical or chemical properties and their biological implications [111]. Ultimately, the process would inform on the rationalise design and synthesis of novel stromal instructive polymers.

Excessive and terminal differentiation of fibroblasts to myofibroblasts has been marked as a defining trait of fibrotic wounds. During late remodelling phase of wound healing, myofibroblasts undergo apoptosis and/or are phagocytised by macrophages. However, recent studies have shown that myofibroblast dedifferentiation to fibroblasts is regulated by macrophage secreted IL-10, and differences in tensile forces in the microenvironment [55], [187]. This has led to development of therapeutics that target myofibroblast dedifferentiation, as means to limit and even reverse fibrosis. Alternatively, a material-based approach involving screening of polymers that instructed dedifferentiation of myofibroblasts to quiescent fibroblasts may present an opportunity to develop

valuable therapeutics (for example, anti-myofibroblast microparticles) for the treatment of pathological fibrosis, such as liver cirrhosis.

The shift from UV free radical polymerised surfaces on the polymer microarray (for high throughput screening) to thermal free radical polymerised polymers (for scaled up studies), presented an opportunity to minimise batch-to-batch variation while providing flexibility in coating different geometrical substrates. Traditional thermal free radical polymerisation applied in this study resulted in quick and efficient polymerisation. However, the technique produced polymers with varying molecular weights and polydispersity. Commercial use (as coatings) of these polymers would require the use controlled thermal free radical polymerisation, such as controlled/living polymerisation, to synthesise polymers with similar chemical properties, thus promoting reproducibility.

To investigate possible molecular cues responsible for the observed differences in cell phenotype and behaviour on the polymers, the thickness of the adsorbed protein layer and the mechanical stiffness of the polymers was studied. Causality was assessed by studying linear regression between polymer properties and observed biological variables (attachment, proliferation, and differentiation) to determine causality. It was determined that differences in fibroblast proliferation and differentiation was not influenced by the thickness of the adsorbed protein over-layer nor the stiffness of the polymers. Thus, it was hypothesised that selective adsorption of serum proteins and/or material-driven activation of adsorbed proteins may elucidate potential mechanisms. Several studies have reported the use of proteomic-based approaches, such as Liquid

Chromatography-Tandem Mass Spectrometry (LC-MS), to identify proteins adsorbed onto polymer surfaces [111], [188]. Furthermore, material-driven activation of adsorbed serum proteins has been extensively studied using an Atomic Force Microscope (AFM) [122], [133], [189]. Future work on these polymers could involve the identification of modulatory mechanisms, using LC-MS and AFM, to inform on polymer and microparticle influenced fibroblast behaviour and phenotype.

Wound healing involves intricate interplay between multiple cell types which orchestrate the healing response. The major cell types involved in directing wound healing are macrophages, fibroblasts, and endothelial cells [19], [46], [63]. This study focused on modulating fibroblast phenotype and behaviour, to regulate the outcome of wound healing. Though, the results presented and discussed showed potential in modulating and accelerating wound healing, it did not account for the cellular complexity observed *in vivo*. A co-culture or tri-culture system between fibroblasts, macrophages, and endothelial cells on the polymers and microparticle scaffolds may help understand the effect of the fibroblast-instructive chemistries on other cell types and overall tissue healing. The proposed study could be conducted in a microfluidic system, thus mimicking circulating monocytes\macrophages, an endothelial cell barrier and resident fibroblasts adherent on the polymer surfaces or microparticles [190].

The scratch wound healing assay used in this study is an efficient, reproducible method of studying cell migration and proliferation, both contributing to wound closure. This study showed that selected hit polymers could not only modulate

fibroblast phenotype, behaviour, and secretion, but also augment the rate of wound closure; thereby accelerating or slowing wound closure. A major limitation of the scratch wound healing model is that it lacks the complexity of the 3D wound bed environment. Advanced 3D wound healing assays have been developed to overcome this limitation. These assays are based on either studying cell migration in a collagen 3D construct or in a human skin equivalent consisting of multiple layers of cells embedded in a hydrogel. Though, these models offer additional levels of complexity, they are not designed to assess biomaterial-based modulation of wound healing. Conversely, wound healing and tissue regeneration models developed by Kollmannsberger et al. (2018) and Wang et al. (2019) offer a degree of adaptability to study polymer modulation in a 3D wound healing model. Further studies adapting these models to investigate polymer augmented 3D tissue healing would provide greater insight. Moreover, a fore mentioned 3D wound healing models could be used to study the effect of the anti-fibrotic and pro-fibrotic microparticle scaffolds in a multi-cellular 3D wound healing model.

The microparticles fabricated and used in this study had a non-biodegradable hexanediol diacrylate (HMDA) core. These were then functionalised with either an anti- or pro-fibrotic surfactant. Studies using these particles served as ‘proof-of-concept’ for the use of anti- or pro-fibrotic microparticles as clinically translatable scaffolds to accelerate and modulate the outcome of wound healing. Though, the anti-fibrotic particles augmented fibroblast phenotype and behaviour towards successful wound healing, the particles were limiting in

applicability due to their non-biodegradable core. Thus, to further enhance applicability, biodegradable microparticles having a poly(lactic acid) (PLA) core instead of an HMDA core have been developed. PLA has been studied to degrade *in vivo* via hydrolysis to form lactic acid monomers, which are removed through renal secretion [192]. Preliminary biological studies using these biodegradable particles have shown similar modulation in fibroblast behaviour.

The clinical applicability of the identified bio-instructive polymers and fabricated microparticles has been pursued by developing bio-instructive dressings in conjunction with an industrial partner, that are aimed at accelerating wound healing. Furthermore, these fibroblast-instructive chemistries can be developed as coatings for implantable medical devices, to alleviate biomaterial-mediated fibrosis. The anti-fibrotic microparticles have shown promise in accelerating wound healing in an *in vivo* diabetic mouse wound model; thus, cementing use as a therapeutic in treating chronic diabetic wounds. Development of biodegradable anti-fibrotic microparticles as injectables or as gels (by combining with an inert cellulose carrier) provides greater translatability.

In conclusion, findings from this thesis demonstrate the ability of surface chemistries to manipulate physiological phenomena: accelerating wound healing and guiding scarred/fibrotic tissue/injuries. This chapter has discussed several future research avenues that would inform on mechanisms of polymer-mediated modulation or would add a level of complexity to the current studies, these are presented in figure 7.1.

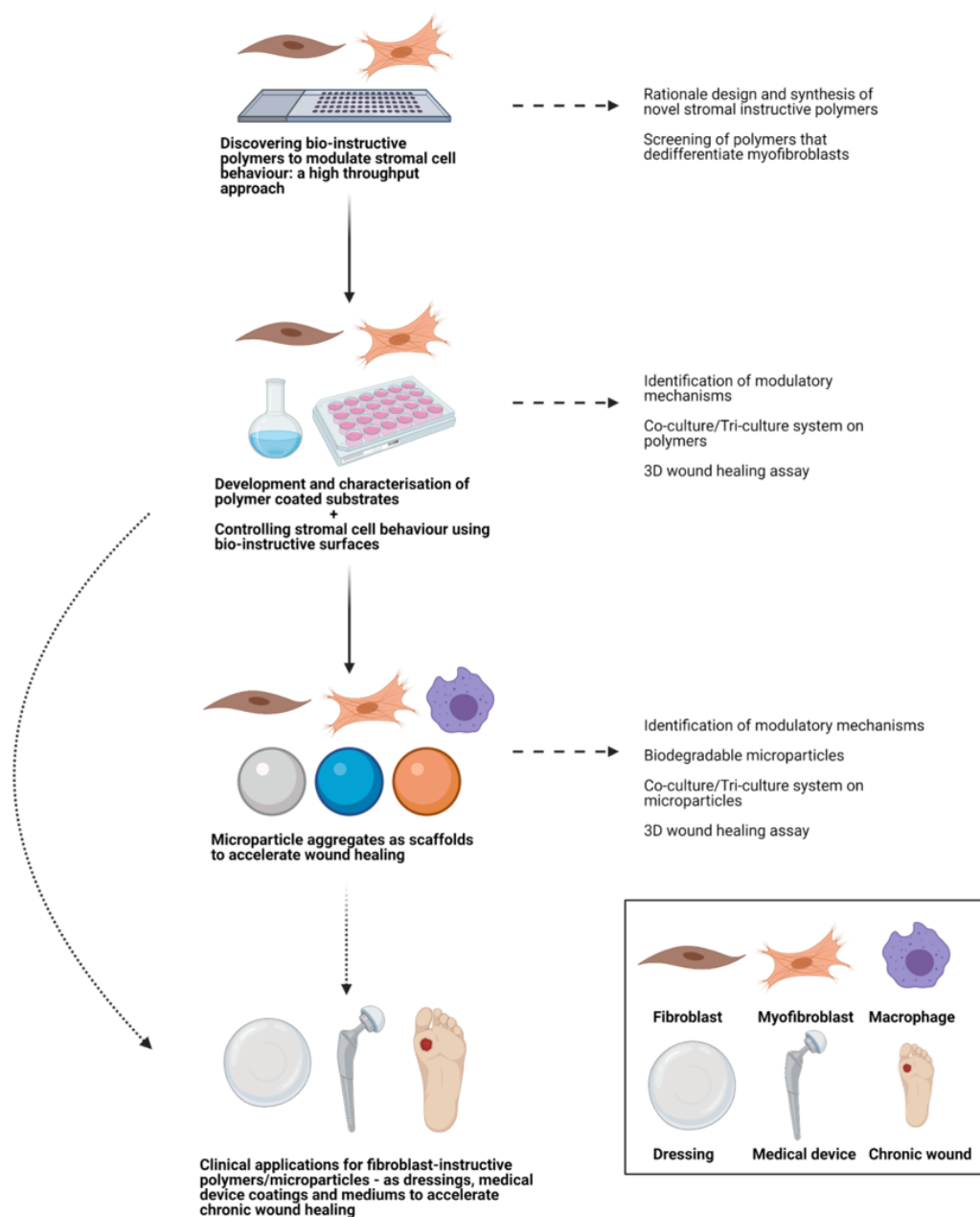


Figure 7.1: Schematic summarising the project, potential research avenues and clinical applications. Figure made in Biorender.com

Chapter 8 - References

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Appendix 1: Polymer library

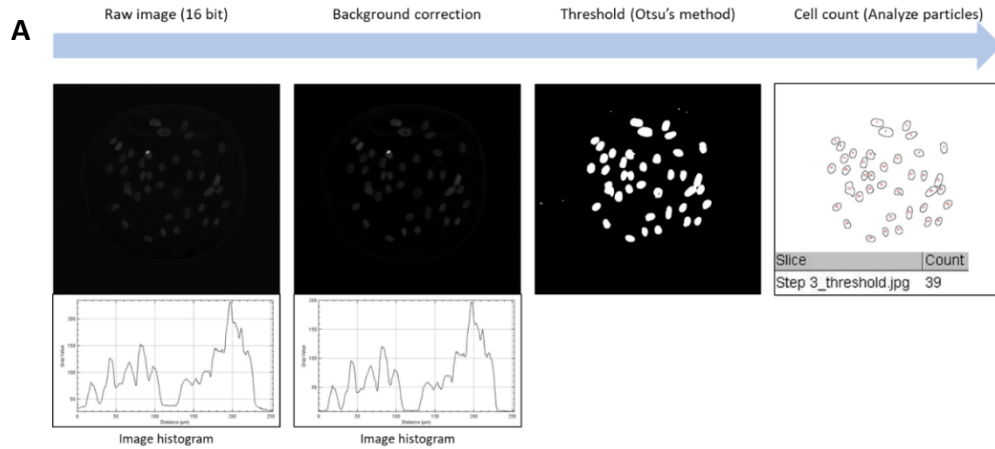
Index	Acronym	Name	Index	Acronym	Name
1	BDDA	Butanediol-1,3 diacrylate	66	iPA	N-Isopropylacrylamide
2	AAcAm	Diacetone acrylamide	67	LaA	Lauryl acrylate
3	AcAPAm	N-[2-(Acryloylamino)phenyl]acrylamide	68	LMA	Lauryl methacrylate
4	ACI	Acryloyl Chloride	69	MAA	Methyl 2-acetamidoacrylate
5	AnMA	Anthracenylmethacrylate	70	MAAH	Methacrylic anhydride
6	BA	Butyl acrylate	71	MAEA	Methacryloyloxyethyl acetoacetate
7	BAGDA	Bisphenol A glycerolate diacrylate	72	MAEACI	[2-(Methacryloyloxy)ethyl]trimethylammonium chloride solution
8	BMAM	N-Benzylmethacrylamide	73	MAL	Methacryloyl-L-Lysine
9	BnA	Benzyl acrylate	74	Mam	Methacrylamide
10	BnMA	Benzyl methacrylate	75	MAPtMA	Methacrylamidopropyltrimethylammonium chloride, N,N'-Methylenebis(methacrylamide)
11	BPEODA	Bisphenol A ethoxylate diacrylate	76	MBMAm	[2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl) ammonium hydroxide
12	BTHPhMA	Benzotriazol-2-yl)-4-hydroxyphenyl]ethyl methacrylate	77	MEDMSAH	N-Methylmethacrylamide
13	BzHPEA	Benzoyl-3-hydroxy-phenoxyl]ethyl acrylate	78	MMAm	Methacryloylamino]propyl]dimethyl(3-sulfopropyl)ammonium hydroxide inner salt
14	CHMA	Cyclohexyl methacrylate	79	MPDSA	Methylthioethyl methacrylate
15	CHPMA	Chloro-2-hydroxy-propyl methacrylate	80	MTEMA	Sodium 3-phenyl-acrylate
16	CMAOE	Caprolactone 2-(methacryloyloxy)ethyl ester	81	NaPhA	Norbornyl methacrylate
17	CNEA	Cyanoethyl acrylate	82	NBMA	N-Dodecylmethacrylamide
18	NBMAM	N-(Butoxymethyl)acrylamide	83	NDMAm	Neopentyl glycol diacrylate
19	DEAEA	Diethylamino ethyl acrylate	84	NGDA	Neopentyl glycol propoxylate diacrylate
20	DEAEMA	Diethylaminoethyl methacrylate	85	NGPDA	2-N-Morpholinoethyl methacrylate, 95%
21	DEGDA	Di(ethylene glycol) diacrylate	86	NMEMA	Naphthyl acrylate
22	DEGDMA	Diethylene glycol dimethacrylate	87	NpA	Naphthyl methacrylate
23	DEGEEA	Di(ethylene glycol) ethyl ether acrylate	88	NpMA	Octadecyl acrylate
24	DEGEHA	Di(ethylene glycol) 2-ethylhexyl ether acrylate	89	ODA	Octafluoropentyl methacrylate
25	DMCSPMA	Dimethylchlorosilylpropyl methacrylate	90	OPMA	Propargyl acrylate
26	DPEPHA	Dipentaerythritol penta/hexa-acrylate	91	PA	Pentaerythritol diacrylate monostearate
27	E3GDA	Triethylene glycol diacrylate	92	PEDAM	Poly(ethylene glycol) methacrylate
28	EA	Ethyl acrylate	93	pEGMA	Poly(ethylene glycol) methyl ether methacrylate
29	EaNIA	Ethyl trans-a-cyano-3-indole-acrylate	94	pEGMEMA	Pentaerythritol tetraacrylate
30	EbCNA	Ethyl-cis-B-cyano-acrylate	95	PETA	Phenyl methacrylate
31	ECNTA	Ethyl-2-cyano-3-(2-thienyl)acrylate	96	PhMA	N-Phenylmethacrylamide
32	EG3DMA	Tri(ethylene glycol) dimethacrylate	97	PhMAm	N-(Phthalimidomethyl)acrylamide
33	EG4DMA	Tetraethylene glycol dimethacrylate	98	PMAM	Poly(propylene glycol) 4-nonylphenyl ether acrylate
34	EGDPEA	Ethylene glycol dicyclopentenyl ether acrylate	99	pPGNEA	Stearyl methacrylate
35	EGDCMA	Ethylene glycol dicyclopentenyl ether methacrylate	100	SMA	Sulfopropyl acrylate potassium salt
36	EGDMA	Ethylene glycol dimethacrylate	101	SPAK	3-Sulfopropyl methacrylate potassium salt
37	EGMEA	Ethylene glycol methyl ether acrylate	102	SPMAK	1,3,5-Triacryloylhexahydro-1,3,5-triazine
38	EGMMA	Ethylene glycol methyl ether methacrylate	103	TAHTA	Tris[2-(acryloyloxy)ethyl] isocyanurate
39	EGPEA	Ethylene glycol phenyl ether acrylate	104	TAIC	Tert-butyl acrylate
40	EGPhMA	Ethylene glycol phenyl ether methacrylate	105	tBA	Tert-butylamino-ethyl methacrylate
41	EHA	Ethylhexyl acrylate	106	tBAEMA	N-tert-Butylacrylamide
42	EHMA	Ethylhexyl methacrylate	107	tBAm	Tert-butylcyclohexylacrylate
43	EOEA	Ethoxyethyl acrylate	108	tBCHA	Tertbutylcyclohexyl methacrylate
44	ExA	Epoxidized acrylate	109	tBCHMA	N-tert-Butylmethacrylamide
45	FMHPNM A	Trifluoro-2'-(trifluoromethyl)-2'-hydroxypropyl]-3-norbornyl methacrylate	110	tBMAM	Tetra(ethylene glycol) diacrylate
46	GA	Glycidyl acrylate	111	TEGDA	Tetrahydrofurfuryl acrylate
47	GPOTA	Glycerol propoxylate triacrylate	112	THFuA	Tetrahydrofurfuryl methacrylate
48	HA	Hexyl acrylate	113	THFuMA	N-(1,1,3,3-Tetramethylbutyl)acrylamide
49	HDDMA	1,6-Hexanediol dimethacrylate,	114	TMBAm	Trimethylcyclohexyl methacrylate
50	HDMPPDA	Hydroxy-2,2-dimethylpropyl 3-hydroxy-2,2-dimethylpropionate diacrylate	115	TMCHMA	Trimethylhexyl acrylate
51	HEA	Hydroxyethyl acrylate	116	TMHA	Trimethoxysilyl propyl acrylate
52	HFCEA	Hafnium carboxyethyl acrylate	117	TMOSPA	Trimethyl propane diallyl ether
53	HFIPA	Hexafluoroisopropyl acrylate	118	TMPDAE	Trimethylolpropane ethoxylate triacrylate
54	HFIPMA	Hexafluoroisopropyl methacrylate	119	TMPETA	Trimethylolpropane propoxylate triacrylate
55	HMA	Hexyl methacrylate	120	TMPOTA	Trimethylolpropane triacrylate
56	HMDA	Hexamethylene diacrylate	121	TMPTA	Trimethylsilyl acrylate
57	HPA	Hydroxypropyl acrylate	122	TMSA	Trimethylsilyl methacrylate
58	HPHMA	N-(4-Hydroxyphenyl)methacrylamide	123	TMSMA	Trimethylsilyloxyethyl methacrylate
59	iBA	Isobutyl acrylate	124	TMSOEMA	Tris(trimethylsiloxy)-silyl propyl methacrylate
60	iBMA	Isobornyl methacrylate	125	TMSOSMA	Trimethylolpropane ethoxylate methyl ether diacrylate
61	iBOA	Isobornyl acrylate	126	TPEMDA	Tri(propylene glycol) diacrylate
62	iBOMAM	N-(Isobutoxymethyl)acrylamide	127	TPGDA	Zinc acrylate
63	iDA	Isodecyl acrylate	128	ZnA	Zirconium acrylate
64	iDMA	Isodecyl methacrylate	129	ZrA	Zirconium bromonorbomanelactone carboxylate triacrylate
65	IOA	Isooctyl acrylate	130	ZrBNCTA	

Index	Acronym	Name	Index	Acronym	Name
131	13BDDMA	1,3-Butanediol dimethacrylate, 98%	196	FuMA	Furfuryl methacrylate
132	14BDDMA	1,4-Butanediol dimethacrylate	197	GDGDA	Glycerol 1,3-diglycerolate diacrylate
133	AA	Allyl acrylate	198	GDMA	Glycerol dimethacrylate
134	AAm	Acrylamide	199	GMA	Glycidyl methacrylate
135	AEMA.C	2-Aminoethyl methacrylate hydrochloride,	200	HBA	Hydroxybutyl acrylate
136	AEMA.C	N-(2-aminoethyl) methacrylamide hydrochloride	201	HBMA	Hydroxybutyl methacrylate
137	AMA	Allyl methacrylate	202	HBOPBA	Hexanediylbis[oxy(2-hydroxy-3,1-propanediyl)] bisacrylate
138	AODMBA	(R)- α -Acryloyloxy- β , β -dimethyl- γ -butyrolactone	203	HDFDA	Heptadecafluorodecyl acrylate
139	AOHPMA	Acryloyloxy-2-hydroxypropyl methacrylate	204	HDFDMA	Heptadecafluorodecyl methacrylate
140	APMAm.C	N-(3-Aminopropyl)methacrylamide hydrochloride	205	HDFHUA	Heptadecafluoro-2-hydroxyundecyl acrylate
141	BAC	N,N'-Bis(acryloyl)cystamine	206	HDMA	1-Hexadecyl methacrylate
142	BACOEa	Butylamino carbonyl oxy ethyl acrylate	207	HEAm	N-Hydroxyethyl acrylamide
143	BAPA	1,4-Bis(acryloyl)piperazine	208	HEMA	Hydroxyethyl methacrylate
144	BAPODA	Bisphenol A propoxylate diacrylate	209	HEODA	Hexanediol ethoxylate diacrylate
145	BDDA	Butanediol diacrylate	210	HFDA	Henicosafuorododecyl acrylate
146	BFEDA	Bisphenol F ethoxylate diacrylate	211	HFHUMA	Hexadecafluoro-2-hydroxy-10-(trifluoromethyl)undecyl methacrylate
147	BHMA	Benzhydryl methacrylate	212	HFPDA	Hexafluoropent-1,5-diyli diacrylate
148	BHMOPhP	2,2-Bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl]propane	213	HMAm	N-(Hydroxymethyl)acrylamide
149	BMA	Butyl methacrylate	214	HMBAM	N,N'-Hexamethylenebisacrylamide
150	BMAOEP	Bis[2-(methacryloyloxy)ethyl] phosphate	215	HMBMAM	N,N'-Hexamethylenebis(methacrylamide)
151	BMENBC	Bis(2-methacryloxyethyl) N,N'-1,9-nonylene biscarbamate	216	HPHOPA	Hydroxy-3-phenoxypropyl acrylate
152	BnPA	Benzyl 2-n-propyl acrylate	217	HPHPBAH	Hydroxyphenyl hydroxyphenylate bis[6-(acryloyloxy)hexanoate]
153	BOEMA	Butoxyethyl methacrylate	218	HPMA	Hydroxypropyl methacrylate
154	BOMAm	N-(Butoxymethyl)acrylamide	219	HPMAm	N-(2-Hydroxypropyl)methacrylamide
155	BPAPGDA	Bisphenol A propoxylate glycerolate diacrylate	220	HPMAP	Hydroxypropyl 2-(methacryloyloxy)ethyl phthalate
156	BPDMA	Bisphenol A dimethacrylate	221	HTFDA	Hexadecafluoro-9-(trifluoromethyl)decyl acrylate
157	CEA	Carboxyethyl acrylate	222	iBuMA	Isobutyl methacrylate
158	CHA	Cyclohexyl acrylate	223	ICEMA	Isocyanatoethyl methacrylate
159	COEA	2-Cinnamoyloxyethyl acrylate	224	MA	Methyl acrylate
160	CzEA	Carbazol-9-yl ethyl acrylate	225	MAAHS	Methacrylic acid N-hydroxysuccinimide ester
161	DAAM	N,N-Diallylacrylamide	226	MAETA	4-Methacryloxyethyl trimellitic anhydride
162	DDDMA	1,10-Decanediol dimethacrylate	227	MAHBP	4-Methacryloxy-2-hydroxybenzophenone
163	DEGMA	Di(ethylene glycol) methyl ether methacrylate	228	MAPU	2-methacryloxyethyl phenyl urethane
164	DFMOA	Dodecafluoro-7-(trifluoromethyl)-octyl acrylate	229	MBAm	N,N'-Methylenebisacrylamide
165	DFHA	Dodecafluoroheptyl acrylate	230	MHMB	Methyl 3-hydroxy-2-methylenebutyrate
166	DFHNMA	Dodecafluoro-2-hydroxy-8-(trifluoromethyl)nonyl methacrylate	231	MMA	Methyl methacrylate
167	DHEBAM	N,N'-(1,2-Dihydroxyethylene)bisacrylamide	232	mMAOEM	mono-2-(Methacryloyloxy)ethyl maleate
168	DIPEMA	2-Diisopropylaminoethyl methacrylate	233	mMAOES	mono-2-(Methacryloyloxy)ethyl succinate
169	DMA	Decyl methacrylate	234	MOPAm	N-(3-Methoxypropyl)acrylamide
170	DMAEA	Dimethylamino-ethyl acrylate	235	MSPMA	Methyldiethoxysilyl-propyl methacrylate
171	DMAEMA	Dimethylamino-ethyl methacrylate	236	NAM	N-Acryloylmorpholine
172	DMAm	N,N'-Dimethylacrylamide	237	NAS	N-Acryloxysuccinimide
173	DMAPA	Dimethylamino-propyl acrylate	238	NBnMA	o-Nitrobenzyl methacrylate, . 95%
174	DMEMAm	N-[2-(N,N-Dimethylamino)ethyl]methacrylamide	239	NDDMA	1,9-Nonanediol dimethacrylate
175	DMMAm	N,N-Dimethylmethacrylamide	240	nOcMA	n-Octyl methacrylate,
176	DMPAm	N-[3-(Dimethylamino)propyl]acrylamide	241	NPhPMA	Nitrophenyl-2-pyrrolidonemethyl acrylate
177	DMPMAm	N-[3-(Dimethylamino)propyl]methacrylamide	242	OFHMA	Octafluoro-2-hydroxy-6-(trifluoromethyl)heptyl methacrylate
178	DOAm	Disperse Orange 3 acrylamide	243	OFPA	Octafluoropentyl acrylate
179	DRA	Disperse red 1 acrylate	244	PAHEMA	Phosphoric acid 2-hydroxyethyl methacrylate ester
180	DVAd	Divinyl Adipate	245	PBBA	Pentabromobenzyl acrylate
181	DVSeb	Divinyl sebacate	246	PDA	1,4-Phenylene diacrylate
182	DYA	Disperse yellow 7 acrylate	247	PDDMA	1,5-Pentanediol dimethacrylate
183	EBAM	N,N'-Ethylenebisacrylamide	248	pEGDA	Polyethylene glycol diacrylate
184	EEA	Ethyl 2-ethylacrylate	249	pEGDMA	Poly(ethylene glycol) (600) dimethacrylate
185	EEMA	Ethoxyethyl methacrylate	250	pEGMEA	Poly(ethylene glycol) methyl ether acrylate
186	EGDA	Ethylene (glycol) diacrylate	251	pEGPhEA	Poly(ethylene glycol) phenyl ether acrylate
187	EMA	Ethyl methacrylate	252	PhEMA	2-Phenylethyl methacrylate
188	EPA	Ethyl 2-propylacrylate	253	PETra	Pentaerythritol triacrylate
189	ETMSA	Ethyl 2-(trimethylsilylmethyl)acrylate	254	pFDA	Perfluorodecyl acrylate
190	F6BA	Hexafluorobutyl acrylate	255	PFPA	Pentafluoropropyl acrylate
191	F6BMA	Hexafluorobutyl methacrylate	256	PFPhA	Pentafluorophenyl acrylate
192	F7BA	Heptafluorobutyl acrylate	257	PFPhMA	Pentafluorophenyl methacrylate, 95%
193	F7BMA	Heptafluorobutyl methacrylate	258	PFPhMA	Pentafluoropropyl methacrylate
194	FDA	Fluorescein O,O'-diacrylate	259	PhA	Phenyl acrylate, 95%
195	FOA	Fluorescein O-acrylate	260	PhEA	2-Phenylethyl acrylate

Index	Acronym	Name
261	PHPMA	3-Phenoxy 2 hydroxy propyl methacrylate
262	PMA	Propargyl methacrylate
263	PMMA	1-Pyrenylmethyl methacrylate
264	pPGA	Poly(propylene glycol) acrylate
265	pPGDA	Poly(propylene glycol) diacrylate
266	pPGDMA	Poly(propylene glycol) (400) dimethacrylate
267	pPGMEA	Poly(propylene glycol) methyl ether acrylate
268	SEMA	2-Sulfoethyl methacrylate
269	tBMA	Tert-butyl methacrylate
270	TBNpMA	Tribromoneopentyl methacrylate
271	tBOCAPAm	N-(t-BOC-aminopropyl)methacrylamide
272	TBPhA	2,4,6-Tribromophenyl acrylate
273	TBPMA	Tribromophenyl methacrylate
274	TCDMDA	Tricyclodecane-dimethanol diacrylate
275	TCSPMA	Trichlorosilyl propyl methacrylate
276	TDFOcA	Tridecafluorooctyl acrylate
277	TDFOMA	Tridecafluorooctyl methacrylate
278	TEGMA	Tri(ethylene glycol) methyl ether methacrylate
279	TFCAm	7-[4-(Trifluoromethyl)coumarin]acrylamide
280	TFPMA	Tetrafluoropropyl methacrylate
281	THMMAm	N-[Tris(hydroxymethyl)methyl]acrylamide
282	TMOBDA	Trimethylolpropane benzoate diacrylate
283	TMOPTMA	1,1,1-Trimethylolpropane trimethacrylate
284	TMOSPMA	Trimethoxysilyl propyl methacrylate
285	tOcAm	N-tert-Octylacrylamide
286	TPhMAm	N-(Triphenylmethyl)methacrylamide
287	VMA	Vinyl methacrylate
288	ZrCEA	Zirconium carboxyethyl acrylate
289	GMMA	Glycerol monomethacrylate
290	MAEP	Monoacryloxyethyl phosphate
291	MAEPC	2-Methacryloyloxyethyl phosphorylcholine
292	PBPhA	Pentabromophenyl acrylate
293	PBPhMA	Pentabromophenyl methacrylate
294	PPPDMA	PEO(5800)-b-PPO(3000)-b-PEO(5800) dimethacrylate
295	CIEA	2-Chloroethyl acrylate
296	MCIMA	Methyl 2-(chloromethyl)acrylate
297	AEAm.C	N-(2-aminoethyl)acrylamide hydrochloride
298	SoIA	Solketal acrylate
299	DHPA	2,3-dihydroxypropyl acrylate
300	PPDDA	3-phenoxypropane-1,2-diyl diacrylate
301	IBESMA	1,7,7-trimethylbicyclo[2.2.1]heptan-2-yl 6-(methacryloyloxy)-4-oxohexanoate
302	tBEMAm	N-(3,3-dimethylbutyl)methacrylamide
303	IPBMA	p-isopropylbenzyl methacrylate
304	2EhMA	2-ethylhexyl methacrylate
305	MbMA	4-methylbenzyl methacrylate
306	CyDMA	cyclododecyl methacrylate
307	CeMA	cetyl methacrylate
308	GMA-AD	geranyl methacrylate
309	LMMA	L-menthyl methacrylate
310	CIbMA	cinnamyl methacrylate
311	NibMA	4-nitrobenzyl methacrylate
312	ClbMA	2-chlorobenzyl methacrylate
313	PiMA	pivaloyl methacrylate
314	DdMA	dodecyl methacrylate
315	MpMA	4-methylpentan-2-yl methacrylate

Appendix 2: Image analysis routines

Algorithm and coding for counting cell nuclei (used for cell attachment and proliferation).



B

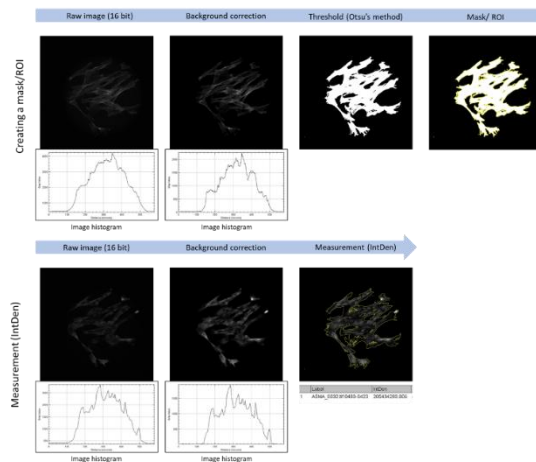
```

1 //Made by Arsalan Latif - Immunology, PhD Research
2 //NOTE: To count number of cells using DAPI in microarray.
3 //Output: Table showing cell count
4
5 path= getDirectory("Choose a directory containing DAPI images");
6 list= getFileList(path);
7 //For loop to apply processing on all images found in folder
8 for(i=0;i<list.length;i++){
9 open(path+list[i]);
10
11 run("16 bit");
12
13 //Gaussian blur - to remove noise.
14 run("Gaussian Blur...", "sigma=3");
15
16 //Background subtraction - to even background
17 run("Subtract Background...", "rolling=15 sliding disable");
18
19 //To Threshold image
20 setAutoThreshold("Otsu dark");
21 //run("Threshold...");
22 setOption("BlackBackground", true);
23 run("Convert to Mask");
24
25 //To segment closely linked nuclei
26 run("Watershed");
27
28 //Cell Counting
29 run("Analyze Particles...", " circularity=0.30-1.00 summarize");
30
31 close();
32 }
33
34 waitForUser("Process Completed");
35
36 //End of for loop

```

A 1: Image analysis routines developed in ImageJ for cell attachment and proliferation. A) Schematic of image analysis routine B) The hard code used. Comments are shown in green.

A



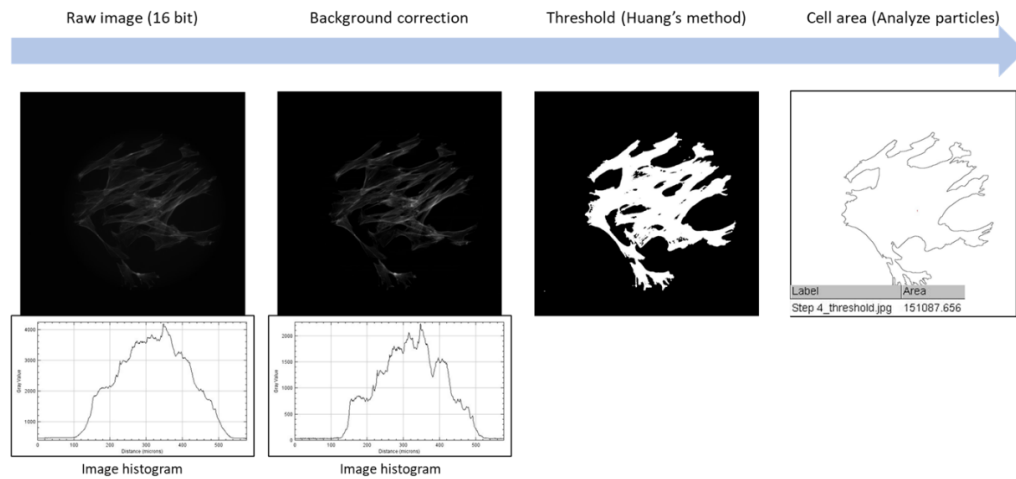
B

```

1 //Made by Arsalan Latif - Immunology, PhD Research
2
3 path= getDirectory("Choose a directory containing Phalloidin images");
4 list= getFileList(path);
5 //For loop to apply processing on all images found in folder
6 for(i=0;i<list.length;i++){
7   open(path+list[i]);
8
9   run("16 bit");
10
11 //Gaussian Blur - with sigma of 2. To smooth image.
12 run("Gaussian Blur...", "sigma=2");
13
14 //Background correction
15 run("Subtract Background...", "rolling=50 sliding disable");
16
17 //To Threshold imaging using Huang method
18 setAutoThreshold("Huang dark");
19
20 //run("Threshold...");
21 setOption("BlackBackground", true);
22 run("Convert to Mask");
23
24 //Creating a ROI based on cytoskeleton
25 run("Create Selection");
26 roiManager("add");
27
28 close();
29 }
30
31 path= getDirectory("Choose a directory containing SMA images");
32 list= getFileList(path);
33 //For loop to apply processing on all images found in folder
34 for(i=0;i<list.length;i++){
35   open(path+list[i]);
36
37   run("16 bit");
38
39 //Gaussian Blur - with sigma of 2. To smooth image.
40 run("Gaussian Blur...", "sigma=2");
41
42 //Background correction
43 run("Subtract Background...", "rolling=50 sliding disable");
44
45 //Applying the ROI made using the phalloidin images
46 roiManager("Select", i);
47
48 //Measuring the IntDen of the cells
49 run("Measure");
50 run("Close All");
51 }
52 IJ.renameResults("Alpha Smooth Muscle Actin Expression");
53 waitForUser("Measurements Completed");
54
55
56 //End of for loop

```

A 2: Image analysis routines developed in ImageJ for measurement of MFI of α -SMA. A) Schematic of image analysis routine B) The hard code used. Comments are shown in green.



```

1 //Made by Arsalan Latif - Immunology, PhD Research
2 //NOTE: Pre-processing and calculating area (through actin staining-Phalloidin) of polymer microarrays.
3 //Output should be a measurement table..
4 //Detailing the total area of cells per image
5
6 //Note to measure certain elements
7 waitForUser("Attention", "Please make sure to have the following selected in set measurements \n Area \n Standard Deviation \n Limit to threshold");
8
9 path= getDirectory("Choose a directory containing Phalloidin images");
10 list= getFileList(path);
11 //For Loop to apply processing on all images found in folder
12 for(i=0;i<list.length;i++){
13 open(path+list[i]);
14
15 run("16 bit");
16
17 //Background subtraction - Rolling ball radius of 70 pixels
18 run("Subtract Background...", "rolling=50 sliding disable");
19 //Remove noise present in images
20 run("Gaussian Blur...", "sigma=2");
21
22 //To Threshold imaging using minimum error method
23 setAutoThreshold("Huang dark");
24 run("Threshold...");
25 setOption("BlackBackground", true);
26 run("Convert to Mask");
27
28 //Measuring area of the cells
29 run("Analyze Particles...", " size=100-infinity summarize");
30
31 close();
32 }
33 waitForUser("Process completed");
34 //End of for Loop
35

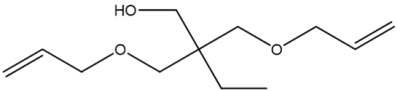
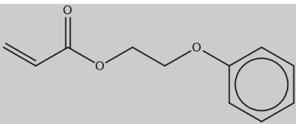
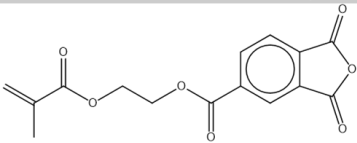
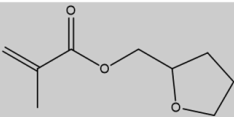
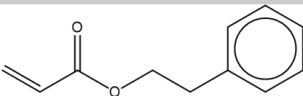
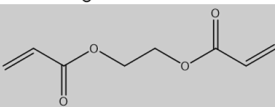
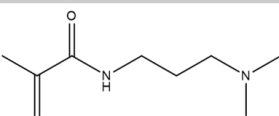
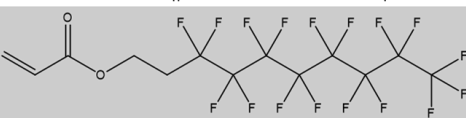
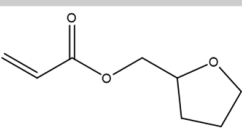
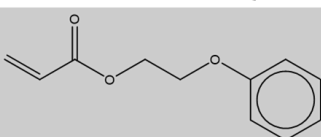
```

A3: Image analysis routines developed in ImageJ for measurement of cell size. A) Schematic of image analysis routine B) The hard code used. Comments are shown in green.

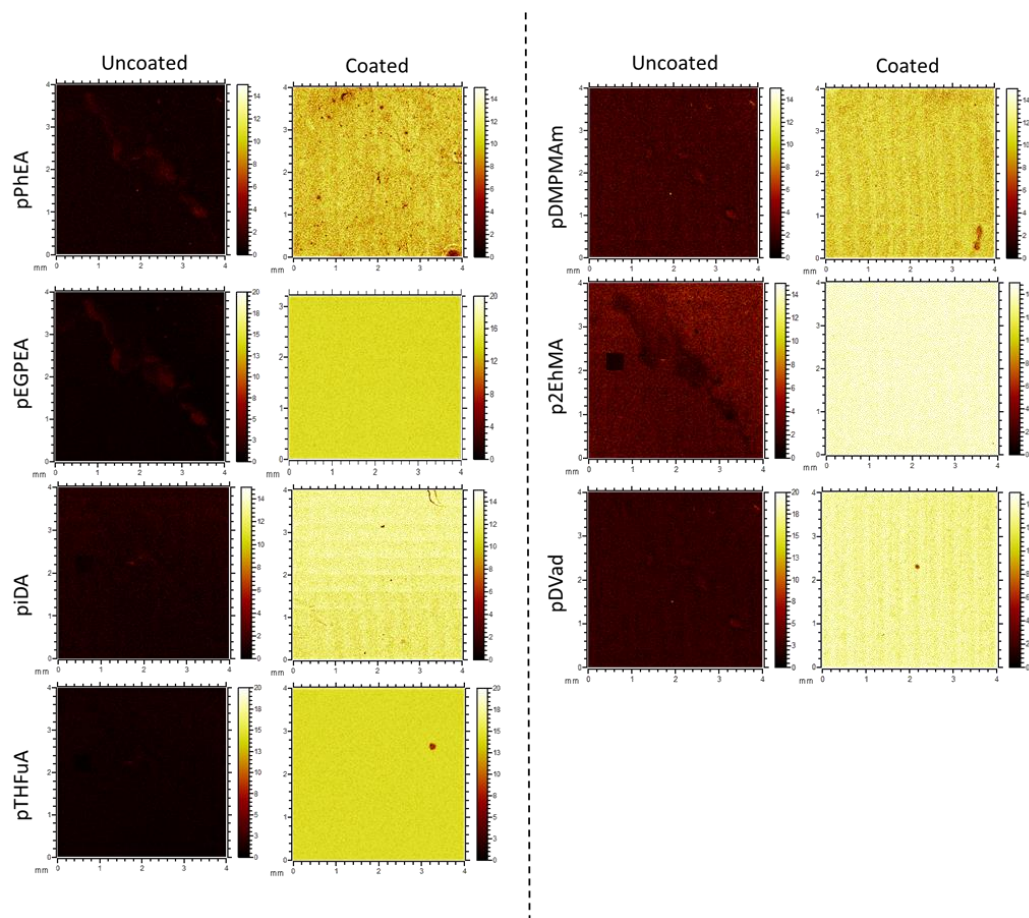
Appendix 3: Supplementary information – Discovering bio-instructive polymers to modulate stromal cell behaviour: a high throughput approach

Table A 1: List of 23 polymers shortlisted based on the cell attachment cell size criteria.

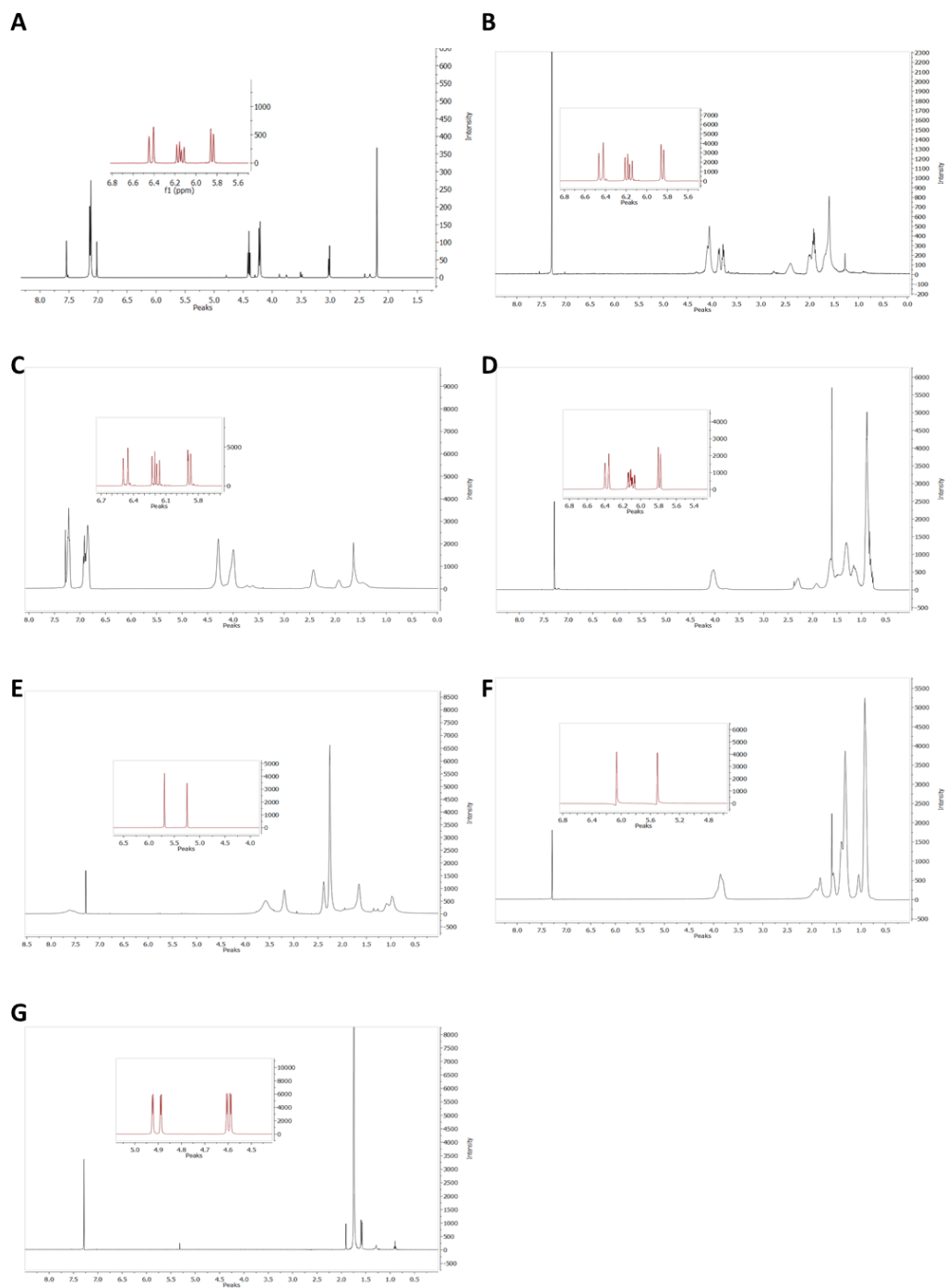
POLYMER	STRUCTURE
2-chlorobenzyl methacrylate (ClbMA)	
L-menthyl methacrylate (LMMA)	
(R)- α -Acryloyloxy- β,β -dimethyl- γ -butyrolactone (AODMBA)	
2-Ethyl hexyl methacrylate (2EhMA)	
Di(ethylene glycol) diacrylate (DEGDA)	
4-nitrobenzyl methacrylate (NibMA)	
IsoDecyl acrylate (iDA)	
Divinyl adipate (DVAd)	
Pentabromophenyl methacrylate (PBPhMA)	
3-phenoxypropane-1,2-diyl diacrylate (PPDDA)	
Ethylene glycol dicyclopentenyl ether acrylate (EGDPEA)	
o-Nitrobenzyl methacrylate (NBnMA)	
Hexafluoropent-1,5-diyl diacrylate (HFPDA)	

Trimethyl propane diallyl ether (TMPDAE)	
Poly(ethylene glycol) phenyl ether acrylate (pEGPhEA)	
4-Methacryloxyethyl trimellitic anhydride (MAETA)	
Tetrahydrofurfuryl methacrylate (THFuMA)	
2-Phenylethyl acrylate (PhEA)	
Ethylene {glycol} diacrylate (EGDA)	
N-[3-(Dimethylamino)propyl]methacrylamide (DMPMAm)	
Heptadecafluorodecyl acrylate (HDFDA)	
Tetrahydrofurfuryl acrylate (THFuA)	
Ethylene glycol phenyl ether acrylate (EGPEA)	

Appendix 4: Supplementary information – Development and characterization of polymer coated substrates

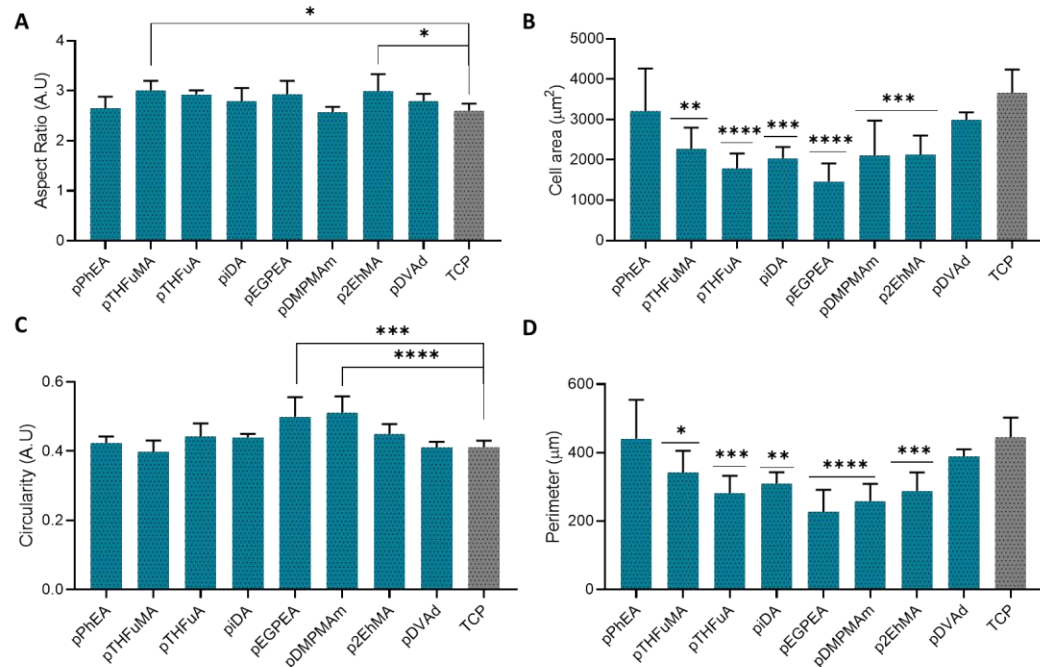


A 4: The ToF-SIMS spectral images of identifier ions of polymers coated on glass coverslips. The images are representative of a 4mm×4mm scan area on the substrate. All polymer coated coverslips showed uniform, hole-free coating.

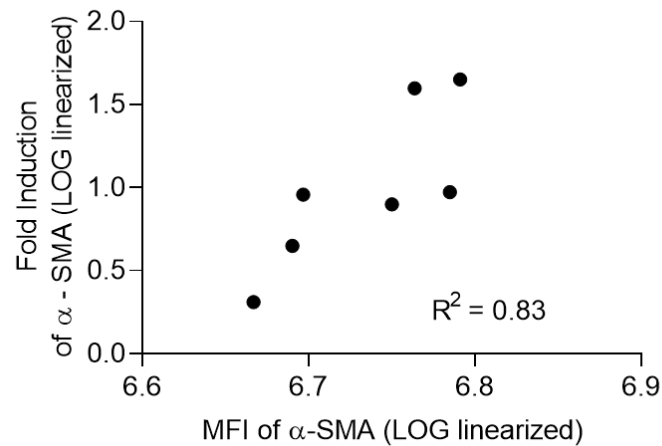


A 5: The ^1H NMR spectra of synthesised polymers. A) Poly(phenyl ether acrylate), pPhEA B) Poly(tetrahydro furfuryl acrylate), pTHFuA C) Poly(isodecyl acrylate), piDA D) Poly(ethylene glycol phenyl ether methacrylate), pEGPEA E) Poly(*N*-[3-(dimethylamino)propyl] methacrylate), pDMPMAm F) Poly(2-ethylhexyl methacrylate), p2EhMA and G) Poly(divinyl adipate), pDVAd.

Appendix 5: Supplementary information – Controlling stromal cell behaviour using bio-instructive surfaces



A 6: Morphometric measurements of fibroblasts cultured on polymers. A) Aspect ratio B) Cell area C) Circularity D) Perimeter



A 7: Correlation plot between SMA expression measured with RT-PCR and image analysis. A high positive R value showed that SMA expression at the gene level was translated into protein expression as measured by the mean fluorescence intensity.