THE INTERPLAY OF MATRIX RIGIDITY AND MECHANICAL STRETCHING FOR THE DEVELOPMENT OF ARTIFICIAL SKIN

BY

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THESIS

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Abstract

Tissue engineering and regenerative medicine provides the possibility for generating organs in laboratorial settings as an off-the-shelf-technology that can provide an alternative therapeutic pathway for transplantations and replace non-functional organs. Many engineered tissues have been attempted in the past decade including skin, blood vessel, cartilage and bone but they lack the mechanical properties and biological functionality of their real counterparts which leads to failure in vivo. Matrix rigidity and mechanical forces are key regulators of tissue homeostasis and are essential for tissue formation, remodeling and healthy function. As a consequence, mechanical stimulation has frequently been employed to improve tissue morphogenesis, and cellular spatial organization but it has not been previously implemented in a synergistic manner with matrix rigidity due to lack of tools in 3D cell culture systems. The goal of this study is to investigate the structural changes, the morphological alterations and tissue formation by developing a novel 3D construct that is resilient with controlled matrix stiffness and which can sustain mechanical strain. This template can be used to further elucidate the biochemical pathways for stem cell differentiation that result into the regeneration of a series of tissues.

This thesis is dedicated to my father,

Vasilis F. Antoniadis

for instilling in me curiosity for science and learning, for being a true believer of the essence of education and thus never questioning -not once in my lifetime- my scores in any test but only caring about my understanding of the fundamentals behind every scientific topic and for showing me through his example that anything is possible to achieve; the only limitations in our lives are those that we impose on.

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Aikaterini Efstathiadou

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Nomenclature

AAD	Adipic Acid Dihydrazide
AFM	Atominc force Microscopy
ANOVA	Analysis of variance
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
HA	Hydroxyapatite
HEPES	4-(2-hydroxyethyl)-l piperazineethane-sulfonic acid
MSC	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2yl)-2,5diphenyltetrazolium bromide
PDMS	Polydimethylsiloxane
Pen-strep	Penicillin-streptomycin
PLA	Poly(lactic acid)
PLGA	Poly(lacticcoglycolic acid)
RGD	arginine-glycine-aspartic acid
SEM	Scanning electron microscopy
SPSS`	Statistical package for the social sciences
Std. Dev	Standard Deviation
TEM	Transmission electron microscopy

Chapter1: Introduction

The development of three-dimensional human tissue models are required in order to expand our understanding of the biological cues within the cellular microenvironment. Tissue engineering needs to successfully mimic the properties and functionality of the in vivo counterparts and provide a novel therapeutic pathway for the development of artificial tissues and ultimately the creation of fully functional artificial transplants. Our goal is to develop a living system technology to restore the microstructure as well as the biological and mechanical properties of degenerated or damaged tissue. Regenerative medicine and tissue engineering rely on the fabrication of scaffolds to investigate the complex morphological organization and geometrical structure of tissues[1]. The mechanical stimulation influences cellular responses such as migration, differentiation and apoptosis and has been used to trigger tissue regeneration in tendons and cartilage[2, 3]. Mechanical stretching can affect the mechanical properties of the substrate and enhance strength, organization and functionality of tissue engineered tissues[4-6]. The conversion of mechanical signal into intracellular biochemical activity, which is known as mechanotransduction, occurs because of external tensile forces and regulatory cues stemming from the cytoskeleton[7]. Mechanical stress and strain in vivo compose the fundamental regulatory mechanical cues that play a key role in cell morphogenesis and the preservation of tissues healthy composition. The strain that cells receive varies from 2D to 3D microenvironments and is dependent on material properties, the state of cell attachment, the ECM stiffness and the external forces applied. Matrix strain can be quantified with physical parameters, or determined by using microscopy and finite element (FE)

techniques. Furthermore, aberration of natural ECM stiffness is known to mediate the pathological responses of several types of diseases. In this study, we developed a method to immobilize alginate hydrogels with varying stiffness on a silicone surface. This allowed us to apply mechanical strain to the hydrogel indirectly by applying the strain to the silicone substrate. In addition, the stiffness of alginate hydrogel could be controlled simply by varying the amount of adipic acid dihydrazide used as a cross-linker. To date, there is limited capacity to independently control elasticity and resilience of a cell-laden matrix while retaining cell viability. Most matrices are readily disintegrated by the cyclic mechanical force, because of their limited resilience. This work will demonstrate a novel methodology to create a 3D construct that is resilient and can become a template to study mechanical cues in biological tissues.

1.1 Effect of the Uniaxial Cyclic Stretch on Cell Biology

Tissue engineering research has been a very promising field for the development of alternative therapeutic pathways in transplantations. Many researchers have explored a bottom-up approach of experimentation in order to create artificial tissues that mimic the properties and functionality of human tissues and ultimately be able to engineer artificial organs from scratch[8]. In addition tissue engineered tissues can become a template for drug testing and for studying the biological cascades that take place in a molecular level in tissue morphogenesis. To date, several researchers have attempted to replicate the complicated cellular microenvironment and form tissue using biomaterials and cell lines but conventional 2D cell cultures have not proven to be an ideal model for this research as they could not develop the

same tissue structure, morphology and functionality as in native tissues. Moreover, cell populations in previous model systems are disorganized and present a variety of shapes. However, cells in vivo are well organized and have a defined and aligned shape. For instance, connective tissues such as the tendon is formed by tendon fibroblasts that can be assumed to be aligned in a parallel manner and exhibit and elongated shape. Therefore, cellular behavior may not ideally replicate the cellular responses in vivo since cellular spatial organization can influence their function.

The mechanical stress and strain are the key regulatory mechanical cues that guide cell morphogenesis and affect the healthy maintenance of tissues and thus ultimately play a role in stem cell fate. Mechanical load affects the tissue dynamics and is triggering cell alignment within the tissue. In addition, mechanical forces exist within the tissue level, when cells are added or removed by cell movements that are associated with morphogenesis, during muscle contraction and relaxation and during bone compression and decompression.

Mechanical condition has been used in experimental settings to study its effect on mechanical properties for engineered tissues like vascular grafts[9]. Under the influence of uni-axial stretching cells are reorienting themselves in a perpendicular direction and their spatial displacement seems to last for several hours after the cessation of the mechanical stimuli. It is remarkable that cells can be influenced by the geometry of their substrate and conform to the different patterns by changing their shape. Substrate topography has been show to play a role in cell structural shape and spreading. Studies have been conducted on this research area and have shown the impact of mechanical stimulation and actin cytoskeleton in cell shape[10-12],

the effects in cell proliferation induced by surface topography[13-15] and the effects in gene expression and protein production[6, 16-20]. Therefore, it is now widely accepted that substrate geometry, and mechanical stretching are playing a key role in cell proliferation, cell adherence, cell alignment, doubling rates and cytoskeleton arrangement.

However, there are several limitations in many of the study models that have been typically used to study the effects of mechanical stretching on tissue morphogenesis. First, there have been shown that cells tend to randomly orient initially and then reorient perpendicularly to the stretching direction when cultured on smooth substrates [21] . As a result, the cells that are subjected to disorganization and realign due to the applied stretching forces are sustaining different rates of surface strains. In addition, cells in vivo are well organized and have controlled cell shape. Conclusively, it is important to develop a system that can efficiently simulate all the above-mentioned parameters and mimic the tissue morphogenesis in vitro.

1.2 Effects of the matrix rigidity on the cellular microenvironment

All types of cells are constantly exposed to external force fluctuations from their cellular microenvironment. A physical signal that plays a crucial role in regulating the cells' fate, their phenotype and behavior is the intrinsic elastic modulus of the extracellular matrix. Cells are modulating their endogenous cytoskeleton contractility which is associated by the forces exerted by the ECM. Conclusively, cells are mechanosensitive and mechanoresponsive to external stimulations and the elastic modulus of their microenvironment through this tensional homeostasis. These balanced forces are playing a key role in the regulation of basic cellular function such as proliferation, adhesion, migration and apoptosis.

Native tissues exhibit a significant differentiation regarding their matrix stiffness and mechanics. In recent years the biophysical nature of the tissue microenvironment along with the biochemical pathways that take place during cell proliferation and adherence to their substrates have been intensely investigated in order to shed light on the development of tissues and ultimately functional artificial organs. It has been found that the relevance of matrix rigidity across many biological tissues has major implication for cell and tissue engineering since it is a feature that can be replicated in vitro settings. Nevertheless, the study of tissue formation of substrates that have a range of stiffness has not been met with standard high throughput models, which in turn limits the systematic research of its effects.

Crosstalk between the ECM and the cell cytoskeleton is a key regulator for all the major biological responses of cells. Cells adhere to their surrounding microenvironment through integrins, the transmembrane receptors, that mediate the signal transduction and interact with the actin cytoskeleton inside the cell. Cell-matrix adhesions or focal adhesions are assemblies through which mechanical force and regulatory signals are transmitted and facilitate the cellular adhesion on the ECM. Focal adhesions serve as the vesicles for the development of mechanical linkages to the sites of integrin binding and clustering.

The stiffness of a material is measured by the elastic modulus which is the mathematical description of a substrate's tendency to be deformed elastically upon application of an external stress. The elastic modulus is thus defined as the slope of its stress-strain curve in the elastic deformation region : $\lambda = \text{stress}/\text{strain}$

where λ (lamda)is the elastic modulus, stress is the restoring force that is created by the deformation divided by the area which the stress force is applied; and strain is the ratio of the deformation caused by stress to the original state of the substrate. The units of λ are pascals.

Native tissues in the body have a wide range of stiffness, from soft brain tissue that has a stiffness of 1 kPa, fat tissue (1-10kPa), striated muscle (10-5 kPa) to stiffer tissues like cartilage (15-20kPa) and precalcified bone (20-100kPa). In contrast conventional tissue culture plastic that is used in vitro have a much stiffer elastic modulus of approximately 106kPa.

The effects of matrix stiffness on tissue formation have been demonstrated on epithelial cells and fibroblasts cultured on collagen-coated polyacrylamide hydrogels. Semler et al[22] have used polyacrylamide (PA) gels to develop substrates with different stiffness for the development of hepatocellular tissue. A number of fabrication approaches have been developed over the years to study cell behavior in microwells and on flexible post arrays which however require very specialized protocols and laboratorial facilities for their development [23-26]. Since then, much research has been conducted by controlling substrate stiffness to show the regulation of the cellular behavior and especially its affect in cell adhesion, cell mechanics and cell-traction forces[27-32].The regulatory cues that are governing cell-matrix interactions have been of great interest in mechanobiology since matrix rigidity and other mechanical external forces influence intracellular signaling, gene expression and cellular biofunctionality. Artificial substrates that possess different density, spacing, topography, geometrical conformation and receptors for cell adherence are necessary in order to gain a better

understanding of the cell-matrix interactions and elucidate the influence of the microenvironment on tissue morphogenesis.

Artificial substrates need to be biocompatible, non-toxic and promote cellular adhesion and proliferation. Cell adhesion peptides incorporated in 2D cell cultures have triggered the proliferation and differentiation of myoblasts and pre-osteoblasts within the artificial ECM microenvironment [33]. The spacing of cell adhesion ligands has also been identified as a factor that can regulate the differentiation of preosteoblasts while its role in 2D and 3D microenvironment is considered ambiguous as its impact in a 3D microenvironment has been questioned in previous studies [34, 35].

1.3 Engineering artificial skin transplants

Skin loss, injury, burn and trauma have been dealt with the use of autologous skin from other parts of the patient's body or the use of xenograft from a different person/cadaver. The former approach might not be efficient due to the lack of skin availability while the latter suffers from the possibility of transplant rejection or infection. Tissue engineered skin has been the product of an off-the-shelf technology where scientists can produce artificial skin from biomaterial scaffolds seeded with the appropriate skin cells. There are several strategies to approach the development of artificial skin, including the culture of epithelial autografts that was first attempted in 1989. Epithelial cells were seeded in vitro and cell sheets were placed on damaged areas to induce would healing and skin regeneration. This procedure has been proven efficient in diminishing immune reactions since the scientists used the patient's own cells [36-

39]. In clinical settings, the efficiency of this method is not promising though due to the dependence of the autografts from the location of the affected area, the incapacity of the epithelial sheets to sustain sheer stress and compression forces, the short viability period and the limited freedom of movements the patient has in fear of autograft disruption [40]. In order to overcome the disadvantages of the cell-sheet skin therapies, dermal-epidermal skin substitutes were engineered with the aim of mimicking the protective functionality of the epidermal skin and the mechanical integrity of the dermis. Prof. Yannas was one of the pioneers in the field and has developed a tissue engineered approach made from collagen scaffold with fibroblasts and keratinocytes [41]. Many approaches of dermal/epidermal substitutes have been developed the past decade that have undergone clinical trials for their efficiency but most of these approaches resulted in morbidity from scarring [42-47]. Although the advantages from the use of the abovementioned dermal/epidermal substitutes are obvious they have not been established as the gold standard since the biomechanical properties are vastly different from the native skin. Therefore, it is obvious that it is of paramount importance to improve the tissue engineered skin so that it can be efficient for clinical use.

1.4 Developing artificial skin technologies through the study of tissue biomechanics

The development of artificial skin relies on the control of the substrate biomechanics and the ability to mimic the biological function of the constituent tissue. For the stabilization of the tissue engineered scaffold biomechanics it is important to use biomaterials that closely mimic the physical and mechanical characteristics of the natural skin components. Collagen type I has been vastly used in artificial skin substitutes due to its natural expression in the human skin, its

biocompatibility, its ability to promote cell proliferation and differentiation. However collagen constitutes a very soft substrate that cannot sustain mechanical forces and is rapidly biodegradable.

In vitro tissue development lacks the alignment and spatial topography of the natural organogenesis. Mechanical stimulation could complement in vitro studies in providing improved tissue morphogenesis and guide lineage switching. Angiogenesis and blood vessel organization can also be enhanced through the use of radial strain [48]. However, despite the variety of studies that shows the role of mechanical stimulation in controlling the mechanical properties of the substrates, it is still not clear what are the underlying biological mechanisms governing tissue morphogenesis and regeneration. Finite element analysis has been implemented to demonstrate the correlation between matrix rigidity and mechanical stimulation in a theoretical level [49].

Extensive research is needed to gain a deep understanding of how external forces are transferred from the substrate to the cells and how the microarchitecture and topography can change this pathway in order to bring in vitro tissue mechanics to the level of natural tissues.

1.5 Objectives of the research project

The proposed work aims at developing a multifunctional 3D construct from immobilized alginate hydrogel onto a silicone substrate (PDMS), as a tissue template where cyclic mechanical strain will be applied during in vitro cell culture with HDF cells for the investigation of the interplay between mechanotransduction and tissue formation.

1.5.1 Specific Aims

Aim 1 Develop a 3D platform with varying stiffness that acts as a tissue engineered scaffold to test the effect of mechanical stress in 2D and 3D cell culture

It is common to build a hydrogel via radical cross-linking reaction, but the silicone-based material acts as a radical scavenger and inhibit the gel formation on its surface. Our aim is to demonstrate a new strategy to chemically link hydrogels of controlled properties with a silicone-based substrate. The mechanical strain will be applied to cells adhered on alginate hydrogel with varying stiffness to explore the combined role of applied mechanical stimulation and mechanical stiffness of ECM on various cellular activities.

Aim 2 Develop a 3D scaffold with controlled alignment and porosity in order to stimuli and direct tissue formation through mechanotransduction

The close an artificial scaffold material mimics the pattern of the living tissue the easier it can promote the natural healing and remodeling processes. We will create an aligned porous network via a novel directional freezing method in order to enhance the supply of nutrient and oxygen of the cells as well as promote intercellular communication and ultimately tissue formation.

Aim 3 Evaluate in vitro the differences in cellular behavior, between human dermal fibroblast cells cultured on smooth and microgrooved substrates, which undergo cyclic stretching

Aim 4 Develop a tissue engineering scaffold for skin regeneration using our novel 3D platform and cell sheet technology

We will develop 3D multi-layered constructs with microtextured patterns made with lithographic techniques and soft embossing. Our hypothesis is that cell morphology and orientation are influenced by the topographical cues on the substrate

1.5.2 Impact of the project

As evident in 2D studies, the nature of the stretch applied (strain range and rate, frequency, uniaxial vs. equiaxial, rest period, etc.), association between cell orientation, alignment and stretch direction, and cell–substrate interaction affect cellular outcomes under stretch. On the other hand during 2D studies, relatively less systematic studies on these parameters have been performed for 3D constructs. ECM constantly undergoes cell-mediated remodeling and diffusion rates of soluble factors and nutrients through the matric are affected following the changes in matrix integrity. The signaling mechanisms for stretch –induced restructuring or remodeling of the tissue microenvironment remain almost elucidated. In addition, the maintenance and development of healthy vasculature through angiogenesis is controlled by a complex interplay between mechanical and biochemical factors. Therefore it is crucial to engineer more robust and biomimetic tissues through the optimization of 3D stretch regimens and to reveal responsible mechanisms and signaling cascades that are triggered physiologically.

1.6 Significance and clinical relevance

In vitro cell culture model systems have been used in combination with cyclic mechanical stretching to study the various biological response that are induced such as cell orientation[21], actin cytoskeletal remodeling[18], changing rates in cell proliferation[50], gene expression and protein synthesis[51]. The development of tissue engineering tools that can elucidate the interplay of matrix rigidity and cyclic tensile stretching for tissue morphogenesis are needed.

1.6.1 The effect of mechanical forces on disease development

Mechanical forces of the cells and their interplay in the different tissues can disturb the cell signaling, known as mechanotransduction and create abnormalities like mutations or misregulation of proteins which in turn are associates with the development of various diseases. Disruption of the force transmission between the ECM, the cytoskeleton and the interior of the nucleus can be the root of a series of conditions such as muscular dystrophies, cardiomyopathy, cancer metastasis and wound scarring. When mechanical forces that are generated within the tissue are altered due to changes in the mechanical properties of the microenvironment, cells functions and behaviors such as apoptosis, survival, proliferation, adhesion and migration may be interfered. The deregulation of the balanced homeostasis between the external mechanical forces and the mechanical forces exerted by the cells has shown to result in diseases of the vasculature, the musculoskeletal system, the liver, the breast tissue and more [52-54]. It is crucial to underline the fact that the forces applied by the elastic modulus of the tissue substrate and the deregulation of the force balance can lead to tumorigenic mutations and cancer progression [55].

In the past decade, it has been observed that tumorigenic microenvironments tend to be stiffer that their surrounding tissues because the interstitial fluid pressure is augmented and the cell proliferative rates are increased[56, 57]. There are critical data which suggest that the decrease in cytoskeletal tension that can be triggered through the reduction of Rho can diminish the tumor size and proliferation as well as the expression of the tumorigenic phenotype [58].

Furthermore, the homeostasis of the cellular microenvironment in epidermal tissues is highly influenced by the disruption from antigens, injuries, burns and abnormal fibroblast proliferation. During cuts and abrasion the surface layers of the skin are damaged, however an incised wound can result in severe bleeding and even encourage the growth of tetanus bacteria and even life-threatening conditions. The body reacts immediately in case of skin disruption by repairing the wound through a healing process where blood vessels reduce blood loss and blood platelets gather at the site to form a clot. Following the blood vessels dilate and cause inflammation with white blood cells starting to clean the site of antigen infection. Collagen layers are produced and capillaries are formed to help skin regeneration. During this process contraction occurs at the edges of the wound and surface skin cells migrate in order to cover the wound and prevent scarring from occurring. However, despite the prompt migration of myofibroblasts to cover the wound and restore the biomechanical integrity of the injured area, scarring may develop and have increased stiffness because of fibrosis [59]. Recently, it has been shown that liver stiffness is an important factor in determining liver fibrosis and is believed to activate the myofibroblast differentiation pathway [60, 61]. Fibrosis can be observed in the vasculature, that often causes reduced cardiac output and augmented cardiac wall thickness [62, 63]. Deregulation of the ECM organization in combination with extensive work load of the

myocardium can result in cardiac failure and necrosis [64]. The balance of the homeostasis in the tissue microenvironment is also important for the prevention of another disease, atherosclerosis, during which we observe hardening of the arteries. A series of substances can build up in the walls of the arteries such as fat and cholesterol and form plaques that narrow the streams where the blood flows. As a result clots may form in these narrow arteries and ultimately starve the tissues from blood and oxygen that may even lead to a heart attack or a stroke. During the progression of this disease, the endothelial cell layer with its anti-adhesive and anti-thrombotic properties is mediating the worsening of the excessive deposition of substances on the blood walls. The mechanical interaction between the endothelium and the basement membrane is a crucial parameter in the preservation of a healthy endothelial barrier [65-68].

Chapter 2: Development of a 3D cell culture template

2.1 Introduction

Mechanotransduction is well recognized as an important regulator of various cellular phenotypes. Mechanical forces are transmitted from extracellular matrices (ECM) to cells through focal adhesion complexes, the intricate sensory machinery involving interaction between cell recognition domains in the ECM and transmembrane receptors and intracellular components of the cell, and illicit specific cellular responses in many physiological and pathological states. The mechanical forces in physiological conditions can be categorized into two types that are combined influence the cellular response: namely, "static" and "dynamic". "Static" force refers to the inherent stiffness of ECM, which varies from tissue to tissue. The stiffness of ECM not only regulates the cellular functions of the residing cells under normal physiological conditions, but is also known to control the proliferation and differentiation of stem cells during developmental stages. Furthermore, aberration of natural ECM stiffness is known to mediate the pathological responses of several types of diseases. "Dynamic" forces are externally generated usually from normal physiological activities, and regulate a variety of cellular functions. For example, pulsatile blood flow from the heart generates cyclic pressure and shear stress onto the vascular system consisting of endothelial cells and smooth muscle cells. Daily physical activities also constantly generate mechanical forces to bone and tissue.

Most of the previous in vitro studies relied on cell culture systems involving elastomeric substrates to apply mechanical stimulation to the adhered cells. However, little attention has been paid to the "static" mechanical environment during the "dynamic" mechanical

stimulation, although it is more physiologically relevant to create a proper extracellular environment matching the physiological tissue stiffness. Furthermore, it would be more desirable to explore the effect of mechanical stimulation while varying the stiffness of the environment. This can be attributed, at least in part, to the lack of in vitro cell culture system which allows one to modulate the stiffness of the cell adhesion substrate.

In this study, we developed a method to immobilize alginate hydrogels with varying stiffness on a silicone surface. This allowed us to apply mechanical strain to the hydrogel indirectly by applying the strain to the silicone substrate. In addition, the stiffness of alginate hydrogel could be controlled simply by varying the amount of calcium ions used as a cross-linker. We applied this technique to immobilize alginate hydrogel onto a silicone-based elastic cell culture plate, and cyclic mechanical strain was applied during in vitro cell culture. The mechanical strain was applied to cells adhered on alginate hydrogel with varying stiffness to explore the combined role of applied mechanical stimulation and mechanical stiffness of ECM on various cellular activities.

In order to study the synergistic effects of mechanical stretching and substrate stiffness we need to develop a novel platform that combines these characteristics, since these factors rarely function alone in the microenvironment . Therefore, the challenge is to assemble a cell-adherent hydrogel with controlled elastic moduli on a stretchable silicone sheet and develop a new chemical methodology to immobilize gel on the silicone sheet. I hypothesize that by chemically conjugating 3D cell laden hydrogel with controlled elasticity to a silicone sheet will allow us to examine the unknown interplay of matrix elasticity and cyclic mechanical stretching.

It is common to build a hydrogel via radical cross-linking reaction, but the silicone-based material acts as a radical scavenger and inhibits the gel formation on its surface. In addition, increasing elastic moduli of a cell-laden hydrogel often leads the gel to be more brittle. Therefore, a stiff gel matrix subject to cyclic mechanical force is readily broken apart, thus aking it difficult to study the role of mechanical force on the cells loaded in a stiff matrix.

In this study we will use alginates that are polysaccharides isolated from brown algae and are capable of forming gels while absorbing 200-200 times their own weight in water. Alginate is a good candidate for this application due to its hydrophilic nature, its pore structure, its interconnectivity, the ability to tune its stiffness and sustain mechanical strain. Polydimethylisiloxane (PDMS) will be the substrate as it has been already used in a wide range of biomedical applications as a result of its physiological innertness, low toxicity, biocompatibility, good thermal stability, low modulus and biomechanical and anti-adhesive properties.

2.2 Methods

2.2.1 Chemical functionalization of the PDMS substrate

PDMS samples were prepared by mixing 10:1 ratio with prepolymer and agent solution and was left to cast overnight in the over at 60C.To each silicon sample, Piranha solution (volume ratio of H2SO4 (70%): H2O2 (30%) = 3: 1) was added and incubated for 30 minutes to functionalize the silicone surface with hydroxyl groups. Then, 3-aminopropyl-triethoxysilane (Sigma Aldrich) was placed on top of the silicone surface and incubated for 1 hour at room temperature to present the silicone surface with primary amine groups. The silicone substrates

were thoroughly washed in deionized (DI) water 3 times with stirring (10 minutes each) in between processing steps.

Separately, 1 wt% alginate (MW ~ 50,000 g mol-1, FMC BioPolymer) solution was prepared in MES buffer (0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) supplemented with 50 mM NaCl, pH 6.5). Sulfonated N-hydroxysuccinimide (Sulfo-NHS, Thermo Scientific) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Thermo Scientific) were added to the alginate solution (molar ratio of alginate: Sulfo-NHS: EDC = 1: 25: 25). Then, 3 mL of the alginate solution was placed into the amine-functionalized silicone-bottomed well, prepared as described above, and incubated for 24 hours at room temperature with gentle shaking (20 rpm).

As a control system, a silicone surface functionalized with poly(ethylene glycol) was prepared. The amine-functionalized silicone was incubated in 1 wt% of α-methoxy-ω-N-hydroxysuccinimidyl ester poly(ethylene glycol) (mPEG-NHS, MW 5000 g mol-1) for 5 hours at room temperature.

As we can see in Figure 1, PDMS backbone consists of silicon and ocide atoms and twomethyl groups that area attached to each silicon atom. In more details, silicone surface was first treated with Piranha solution to present hydroxyl groups (I), followed by silanization with 3-aminopropyltriethoxysilane to present primary amine groups (II). Alginate was finally conjugated to the silicone via amide-forming EDC coupling (III).

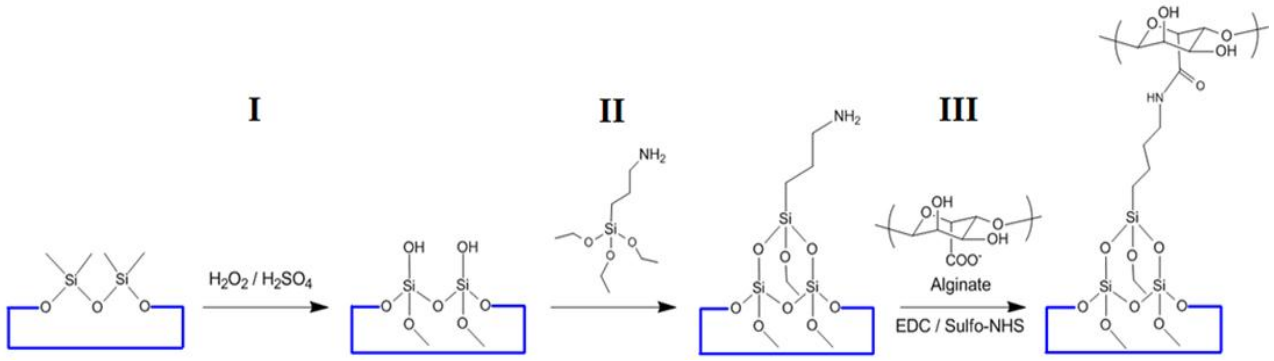


Figure 1 PDMS Chemical modification treatment

AAD-alginate mixture was placed on top of alginate-functionalized silicone surface, and allowed to form the hydrogel.

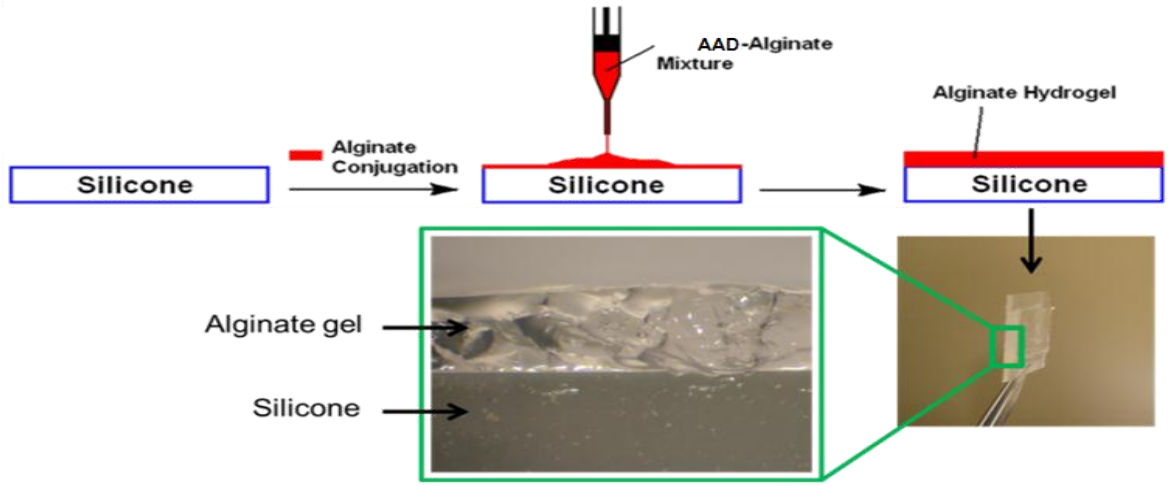


Figure 2 Experimental scheme of the hydrogel immobilization onto the PDMS substrate

2.2.2 Hydrogel Synthesis

Chemical crosslinking has been achieved with carbodiimide chemistry and the use of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) that assists in increasing strength and lowering degradation levels [69]. This method provides enzymatic stability and the capability to tune the biomaterial's stiffness by increasing the concentration of EDC [70] .

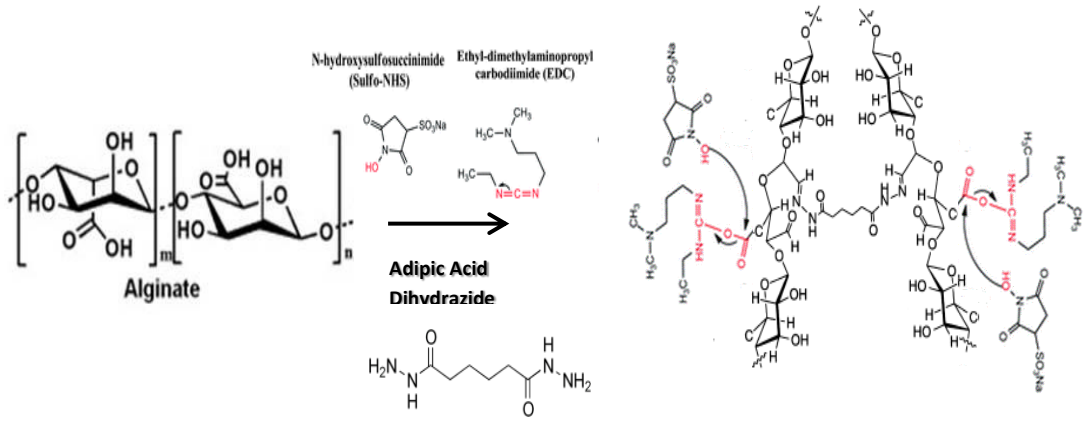


Figure 3 Chemical reaction of the AAD/aldinate hydrogel

Low molecular level alginate was dissolved in MES buffer at 1wt%. Then, Sulfo-NHS, EDC and Adipic Acid Dihydrazide were added to the presolution to form a gel.

2.2.3 RGD peptide conjugation to alginate

Alginate was dissolved in the MES buffer at 1 wt%. Then, Sulfo-NHS, EDC, and the peptide GGGGRGDSP ("RGD peptide", Mimotopes Pty Ltd.) were added to the alginate solution, and stirred for 24 hours (molar ratio of alginate: Sulfo-NHS: EDC: RGD peptide = 1: 3: 2: 2). The solution was dialyzed against DI water for 2 days, replacing the water twice per day. The dialyzed RGD peptide conjugated alginate (RGD-alginate) solution was lyophilized, and reconstituted to a 2 wt% solution.

2.2.4 RGD-alginate hydrogel fabrication on alginate functionalized silicone surface

0.5 mL of RGD-alginate solution, prepared as described above in Section 2.2, was placed into a syringe, and varying amount of calcium sulfate slurry (0.1 g mL-1 in water) was placed into another syringe. The two syringes were connected with a Luer-Lok connector. The two solutions were mixed thoroughly by rapidly moving the solutions between two syringes 10 times. The resulting mixture was placed on top of a silicone-bottomed well functionalized with

alginate, and a cover slip (20 mm × 20 mm) was placed on top of the mixture. The mixture was then incubated at room temperature for 1 hour, resulting in a thin alginate hydrogel layer. The coverslip was then removed, and the hydrogel was washed with DI water.

2.3 Results

2.3.1 Mechanical properties of alginate hydrogels on silicone surface

The maximum strain of silicone substrate, above which the alginate hydrogel detaches from the silicone, was measured by uniaxial stretching. The silicone substrate attached with alginate hydrogel was cut into a rectangular shape (1 cm × 2.5 cm), and the both edges of the silicone were gripped onto a mechanical testing system (MTS Insight). Then, the silicone was continuously stretched at a rate of until the hydrogel detached from the silicone.

The hydrogel stiffness was evaluated with measurement of the elastic modulus of a hydrogel. The alginate hydrogel was cut into disks with a diameter of 10 mm. Each hydrogel disk was compressed at a rate of 1 mm min-1 using a mechanical testing system (MTS Insight). The elastic modulus was calculated from the slope of a stress vs. strain curve at the first 10% strain.

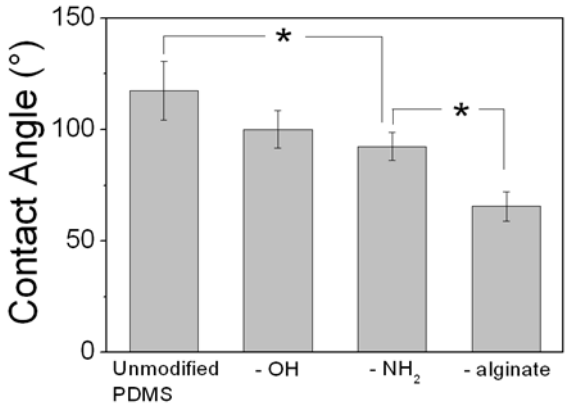
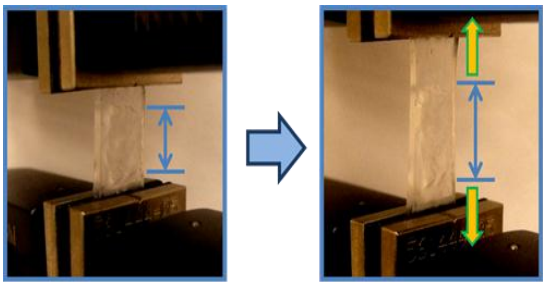


Figure 4 Contact angle measurements of PDMS (unmodified, presenting -OH groups, -NH2 groups and with alginate layer on top



Modulus (kPa)	Maximum strain (%)
3	37.0 ± 4.2
15	27.5 ± 3.8
42	21.3 ± 2.5

Figure 5Maximum strain of alginate hydrogel adhered to silicone substrate from uniaxial stretching

In order to test the adherence of the PDMS-hydrogel interface we performed a delamination limit experiment where we have developed n=15 samples of the three conditions that are shown in the Figure 6 below. In condition A the PDMS has been fully functionalized and has been treated with APTS and an alginate layer on top using EDC/NHS chemistry. In condition B the PDMS has been treated with APTS only and in condition C we have used bare PDMS.

Figure 6 PDMS sheets with circular hydrogels deposited on top and different types of substrate functionalization. A. PDMS fully functionalized B. PDMS treated with APTS C. Bare PDMS

We have used a stretching unit with an adjusted high-resolution camera and we manually applied stress to quantify the breaking point or delamination point of the interface. Average substrate strain was calculated by the DIC analysis. To measure the strain field of the PDMS substrate experimentally we have put markers on the substrate surface and stretch was applied incrementally. Following, strain was calculated using the built in digital image correlation (DIC)

function in Matlab that locates multiple traceable points with each marker and measures their displacement through sequential images.

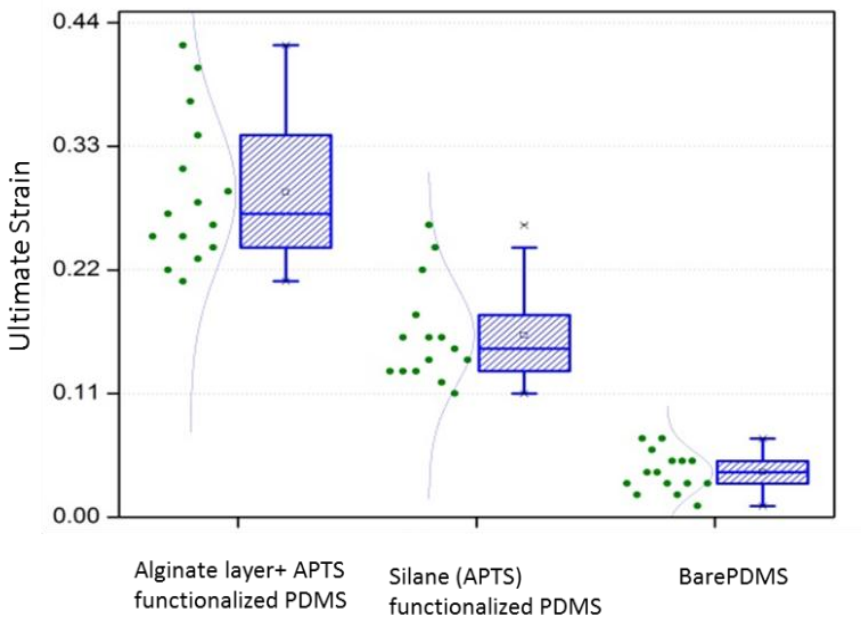


Figure 7 Ultimate strain of the interface between the PDMS and the immobilized hydrogel for different functionalizations

As shown in Figure 7, the ultimate strain which is considered to be higher in statistically significance for the fully functionalization method proving how strongly the interface can adhe re.

AAD/uronic acid	Modulus (kPa)	Interfacial strain
0.0014	3	0.15
0.0019	15	0.21
0.0027	42	0.27

Figure 8 Elastic modulus and interfacial strain of the PDMS/hydogel with dfferent crosslinking densities

2.4 Conclusions

Silicone surface was chemically modified with alginate following a series of modification steps in order to attach calcium-crosslinked alginate hydrogel on the silicone. Silicone was first oxidized to present hydroxyl groups using H2SO4/H2O2 ("Piranha solution") . Following the oxidation, silanization with 3-aminopropyltriethoxysilane presented the silicone surface with primary amine groups. Finally, alginate molecules were conjugated to the silicone by the reaction of amine groups on silicone with carboxylic groups of alginate via amide-forming carbodiimide coupling. Separately, poly(ethylene glycol) (PEG) was conjugated to the silicone surface as a control. The modification steps were monitored by measuring the contact angle of water droplets on the silicone surface. The contact angle of untreated silicone was 120°. Subsequent modifications steps resulted in the significant decrease of contact angle, due to the increasing hydrophilicity of the silicone surface imparted by hydrophilic functional groups.

AAD cross-linked alginate hydrogel was immobilized on the silicone surface, by allowing the gelation on top of alginate-functionalized silicone surface. Alginate can form interconnecting network to form hydrogel by interaction with divalent ions such as calcium ions. The alginate molecules on the silicone surface would participate in the cross-linking reaction between alginate and calcium ions, thus forming a bond between the hydrogel and the silicone. Alginate hydrogel indeed was attached to the alginate-functionalized silicone surface. On the other hand, alginate hydrogels that was formed on unmodified silicone surfaces immediately came off the surface, likely due to the non-wettable hydrophobic surface of silicone. Alginate hydrogels also did not adhere to the silicone substrates with hydroxyl and amine groups, and

that functionalized with poly(ethylene glycol), which shows the alginate hydrogel could not bind to even more hydrophilic and wettable silicone surface, and proves that alginate on silicone surface is involved in the adhesion of alginate hydrogel.

Next, the stiffness of alginate hydrogels was controlled by varying the amount of AAD as a cross-linker, while keeping the concentration of alginate was kept constant at 2 wt%. Increasing the concentration of AAD from 0.1 M to 0.4 M resulted in the increase of elastic modulus from 3 to 40 kPa. Further increase in calcium ions did not result in increased modulus, indicating that alginate molecules have fully reacted with AAD to form elastically responsive cross-links.

Alginate hydrogel must be able to deform accordingly without losing its structural integrity to the external mechanical stimulation mediated by silicone substrate to which the hydrogel is attached. Thus, the maximum strain of alginate hydrogel attached to a silicone surface from uniaxial stretching was evaluated. The alginate hydrogel was able to withstand significant mechanical deformation while remaining attached to the silicone surface. However, the maximum strain of alginate hydrogel decreased with increasing hydrogel stiffness, as stiffer hydrogels detached from the silicone surface more readily. This is due to stiffer hydrogels resisting structural deformation, thus causing more shear stress at the hydrogel-silicone interface.

Chapter 3: Frequency and strain of cyclic tensile stretching enhances artificial skin rigidity

3.1 Introduction

The past year there were more than half a million patients with skin burn injuries that were hospitalized, with the lower degree burns being treated with wound dressings and split injuries[71]. However, extended burn injuries that cover the majority of the human body are very challenging and need an alternative therapeutic approach to be successfully treated. Tissue regeneration is a promising pathway that can produce approximately 16.5 times larger skin sheets from donor skin than any other method. Current engineered skin constructs cannot mimic the ultimate tensile strain, elastic modulus and surface topography of the natural skin and thus it is imperative to develop a novel approach with improved biomechanics [72-75].

Mechanical forces are influencing the normal skin physiology and can be applied externally and internally [68, 76] . There have been studies that showed that mechanical stretching can induce the increase of tensile strength in engineered skin and assist cellular alignment and scaffold architecture.

In this current, we expose our 3D construct to cyclic mechanical strain and assess the mechanical properties and tissue organization.

3.2 Methods

3.2.1 Development of microporous network in hydrogels for 3D cell culture

In most scaffolding materials, the microporous network of the substrate can substantially contribute in spatial organogenesis, and tissue alignment. Anisotropically aligned micropores are often essential in order to allow homogenous cell distribution and interconnection throughout engineered tissues. Finally, increased porosity also improves the diffusion of nutrients and oxygen, a parameter which is very crucial in scaffolds especially in the absence of a functional vascular system.

Therefore, we have developed a freeze-drying technique which enhances the porous directionality as shown in the figure be low.

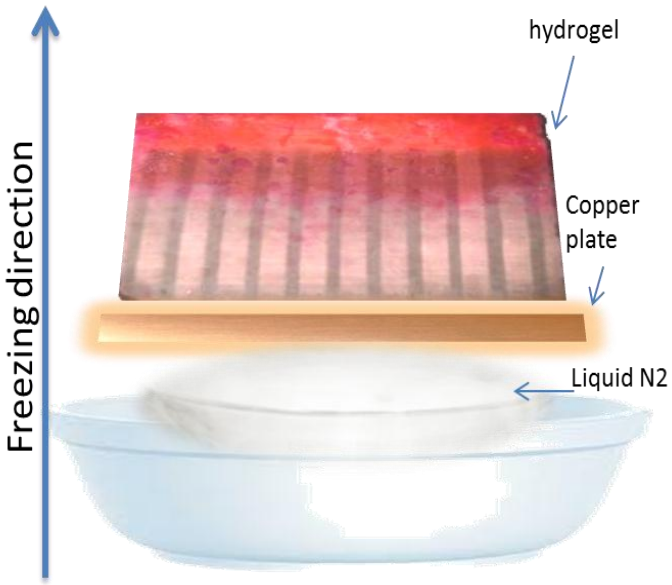


Figure 9 Freeze-casting technique for increased microporosity of the hydrogels

More precisely, we developed alginate gels crosslinked with AAD that have 1mm thickness and shaped in as circular discs. The hydrogel disc is deposited on top of a Copper (Cu) plate of 0.05mm thickness that acts as a thermoconductor. The extremely low temperature of liquid Nitrogen that is below the plate (-196C) results in the rapid formation of ice nuclei and the growth of small ice crystals. Consequently, the samples are introduced into the lyophilizer, a chemical pump/vacuum chamber, where the surrounding pressure is reduced and allows the frozen water in the material to sublimate directly from the solid phase to the gas phase.

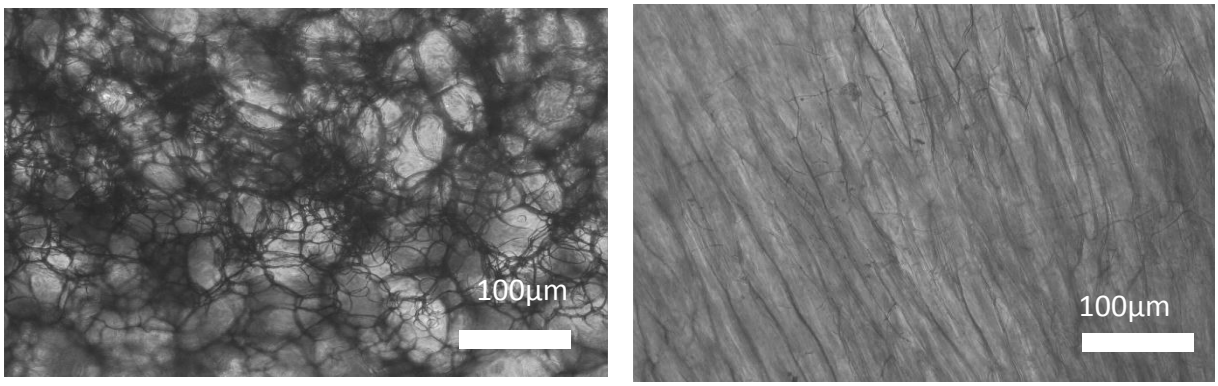


Figure 10 Bulk porous network (left) and aligned porous network after freeze-casting (right)

In addition, we have tested the porosity of the AAD/alginate gel with 5% and 20% crosslinking density and with the presence or absence of RGD. RGD sequence is recognized by cell-surface receptors, such as integrins, which are known to mediate cell adhesion. About half of the 24 integrins that bind to the ECM molecules are adhering in an RGD sequence dependent manner. As a resut conjugation of RGD

molecules to inorganic or polymeric surface has been shown to promote cell adhesion.

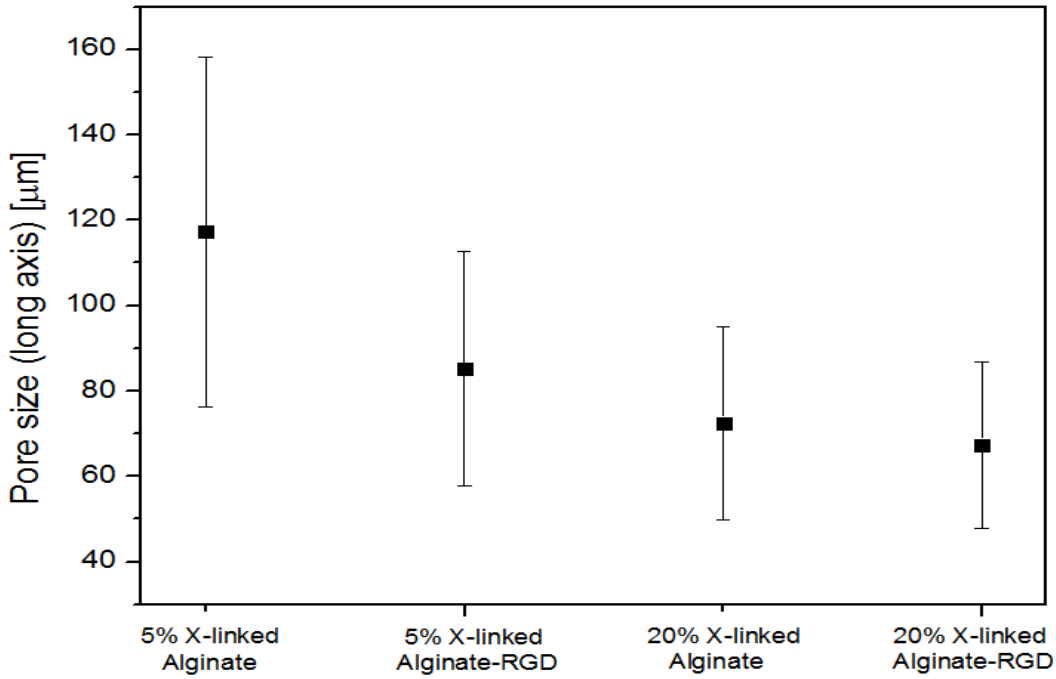


Figure 11 Range of porous diameter in alginates with different crosslinking densities

3.2.2 Effect of cyclic mechanical stretching on the hydrogel/PDMS sheet

For this study we have used a custom made cyclic mechanical stretching unit that has a mechanical motor for controlling the frequency and strain.

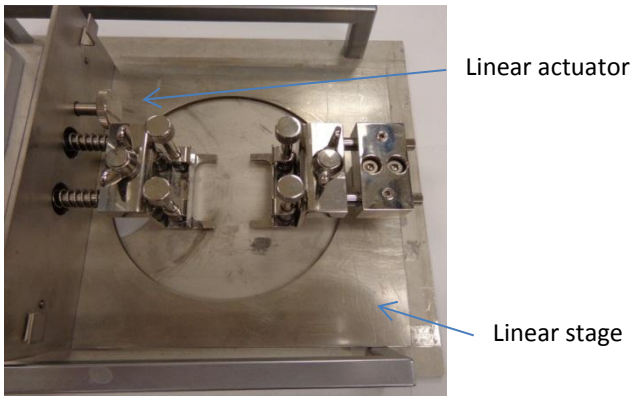


Figure 12 Linear mechanical stretching unit with controlled frequency from 0.1-1Hz and strain 0.1-0.3

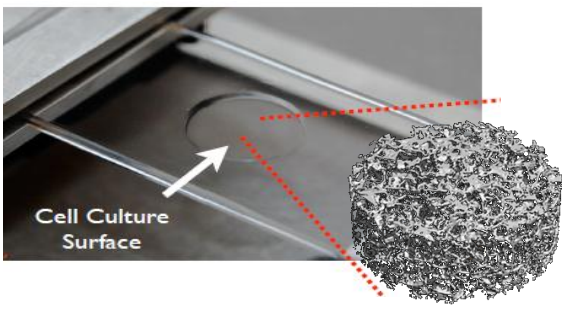


Figure 13 PDMS/hydrogel construct mounted on the stretching unit

As shown in Figure 13, the PDMS is 1mm thick and has a circular microgrooved area that is functionalized so that the circular hydrogel disc is adhered. This construct is mounted on the unit by the two edges.

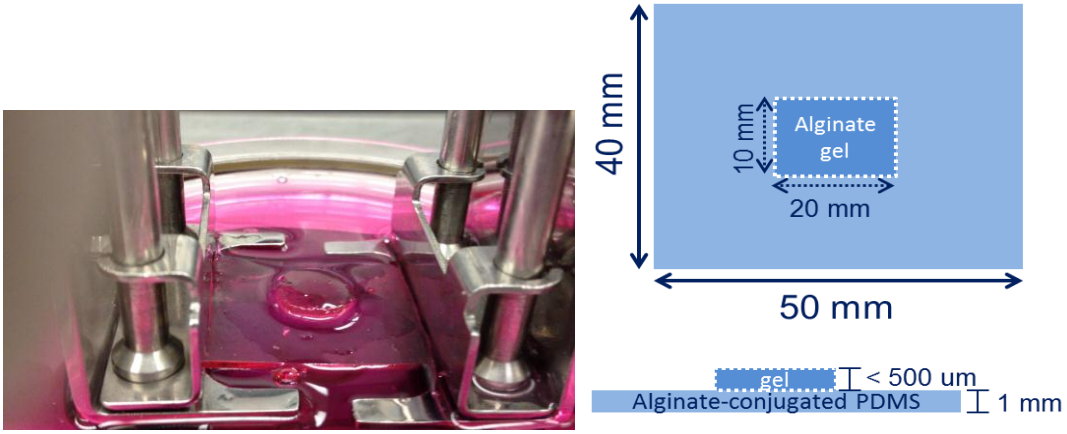


Figure 14 Experimental setup of the 3D cell culture construct on the mechanical stretching unit in sterile conditions and the presence of media.

As shown in Figure 14, the substrate deformation is transmitted onto the adherent gel that has been seeded with cells and this system allows high resolution imaging for the observation of the morphological changes of the 3D cell culture.

3.2.3 Analysis of viability and proliferation of cells encapsulated in hydrogels

NIH3T3 fibroblasts (purchased from the American Type Culture Collection) were cultured in passage 6 and seeded on top of the gel in a droplet manner at a density of 1×10^{6}cells mL^{-1} . In order to promote cell adhesion RGD sequence was incorporated in the prepolymer mix. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation, Calrsbad, CA) and 100 units/ml penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA) at 37°C in a 5% C02 incubator. The cell culture medium was changed every other

day. In order to examine the effects of hydrogel stiffness on cell proliferation both high and low molecular weight alginates were used and the AAD/uronic acis ratio was varied to achive hydrogels with 5kPa, 25kPa and 40kPa. Gel disks were punched using a 5 mm diameter puncher and were consequently incubated in DMEM overnight. Cell numbers on the 2D gels were quantified on Days 1, 3 and 5 from 5 disks from each condition. The cell counts were normalized by the samples counted on Day 1.

Cell viability was assessed with MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at various time points. MTT is absorbed enzymatically and reduced by the live cells to transform in MTT formazan. The samples were scanned to measure their absorbance at 570nm on the 1,3,5,7,10 day. In brief, each hydrogel disk was placed in one well of a 96 well plate with 0.1mL of media. 10µL of MTT Reagent (ATCC) is then distributed to each well and left for 4 hours at 37°C. Following, 0.1mL of stop solution was added, which is made out of 20% sodium dodecyl sulfate in water/dimethylformamide in a 50:50 ration), and the samples were incubated overnight to let it diffuse throughout the hydrogel. The samples were scanned at 570nm using a spectrophotometer (Synergy HT, BioTek), which is the wavelength that shows the number of living cells that are positively stained with MTT. We normalized the outcome by subtracting the absorbance of normal media and then the normalized absorbance was divided by that measured right after cell encapsulation in order to acquire the cell viability as a percentage rate. The absorbance was observed for 10 days to provide evidence for cell proliferation rate over time, while morphological changes and cellular divisions were observed under a LEICA DMIL microscope.

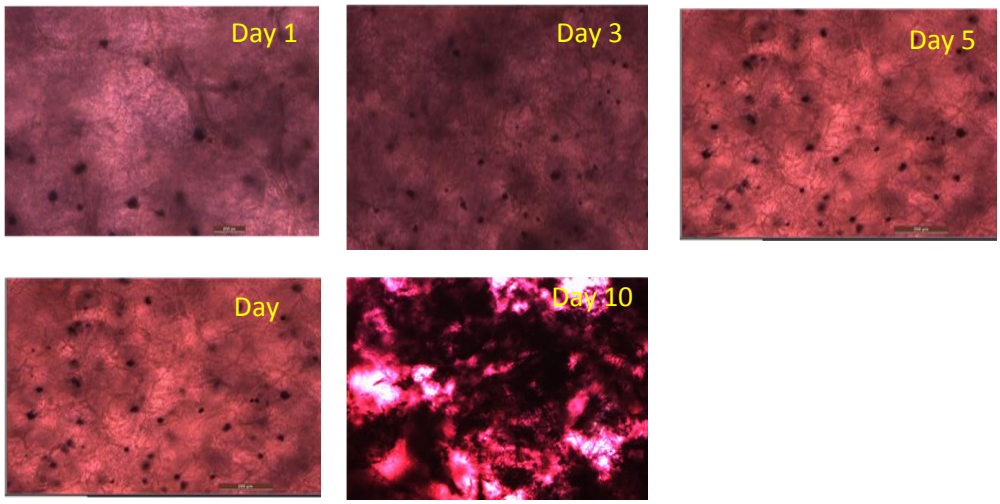


Figure 15 Phase-contrast microscopi images of cells takenon Day 1,3,5,7,10. The viable cells were positively stained with MTT (dark purple) (Scale bar:100μm).

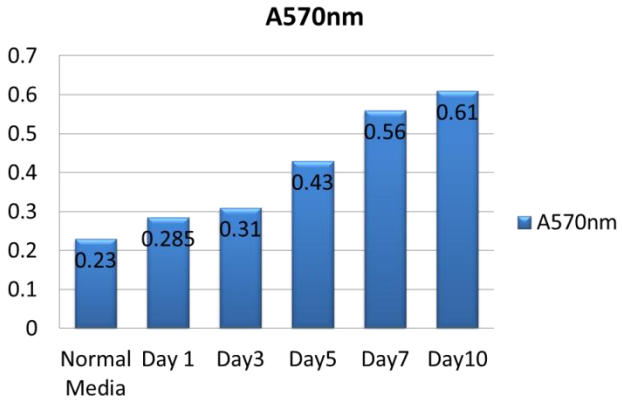


Figure 16 The amount of live cells in the hydrogel was quantified by MTT using the spectrophotometer at 570nm absorbance.

3.2.4 Characterization of stiffness and swelling ratios of hydrogel substrates

AAD/alginate hydrogels were tested for their stiffness by calculating their elastic modulus. The hydrogels were formed and punched into circular disks of 1cm diameter and were incubated in PBS for 24h at 37°C. The disks were put under uniaxial compression at the mechanical testing system MTS (Insight, MTS systems) at a rate of 1mm.min. The Young's modulus was calculated from the slope of stress (σ)vs strain (λ) at the first 10% strain.

Moreover, the hydrogel disks underwent swelling ratio testing by measuring the weight ratio of a swollen hydrogel that had been incubated in PBS for 24h at 37°C to a dried gel.

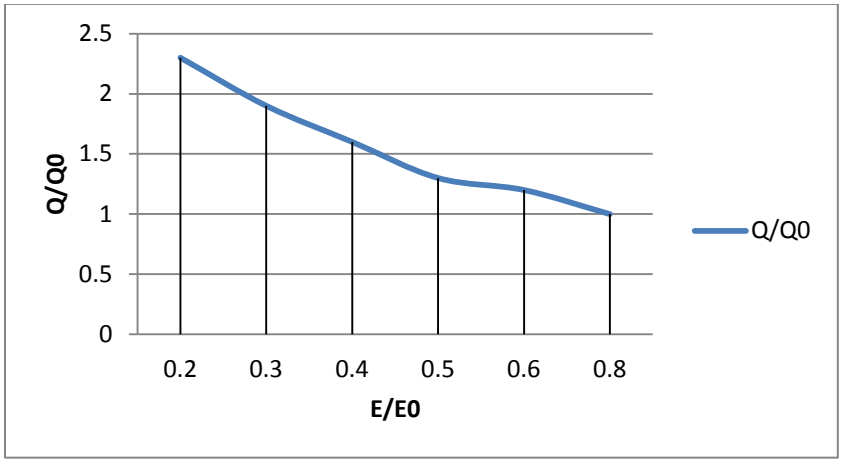


Figure 17 The inverse dependency of normalized swelling ratios (Q/Q0) on the normalized elastic modulus (E/E0)

3.3 Results

Our hydrogel system can be used as a cell encapsulation and transplantation device in various biomedical applications. Since cells are mechanosensitive and mechanoresponsive, the physical and mechanical properties of the hydrogels can regulate a variety of functional and behavioral cellular activities. In our system we are able to tune the matrix rigidity, contact angle, resistance to fracture and interaction with molecules and cells.

In order to test the interdependency of the biomechanics of our system with cellular spreading and spatial displacement, we have used out mechanical stretching unit and applied mechanical stimulation on a 2D cell culture. We have used NIH3T3 cells (P9) that were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation, Calrsbad, CA) and 100 units/ml penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA). We developed

10 samples of functionalized PDMS substrates (as describes in the previous chapter) and immobilized AAD/ alginate-RGD of 40kPa elastic modulus on top of it, that didn't undergo the lyophilization process. Uniaxial cyclic stretching was applied at 1Hz frequency and 0.1 strain for 20min/3x/day over a period of a week. We seeded 10^4 cells per disc by using the hanging drop method. The culture was observed through optical microscopy and pictures were taken daily to monitor morphological changes of the samples. Following the images were analyzed using the software ImageJ and we correlated the before and after maps as shown in Figure 18 below.

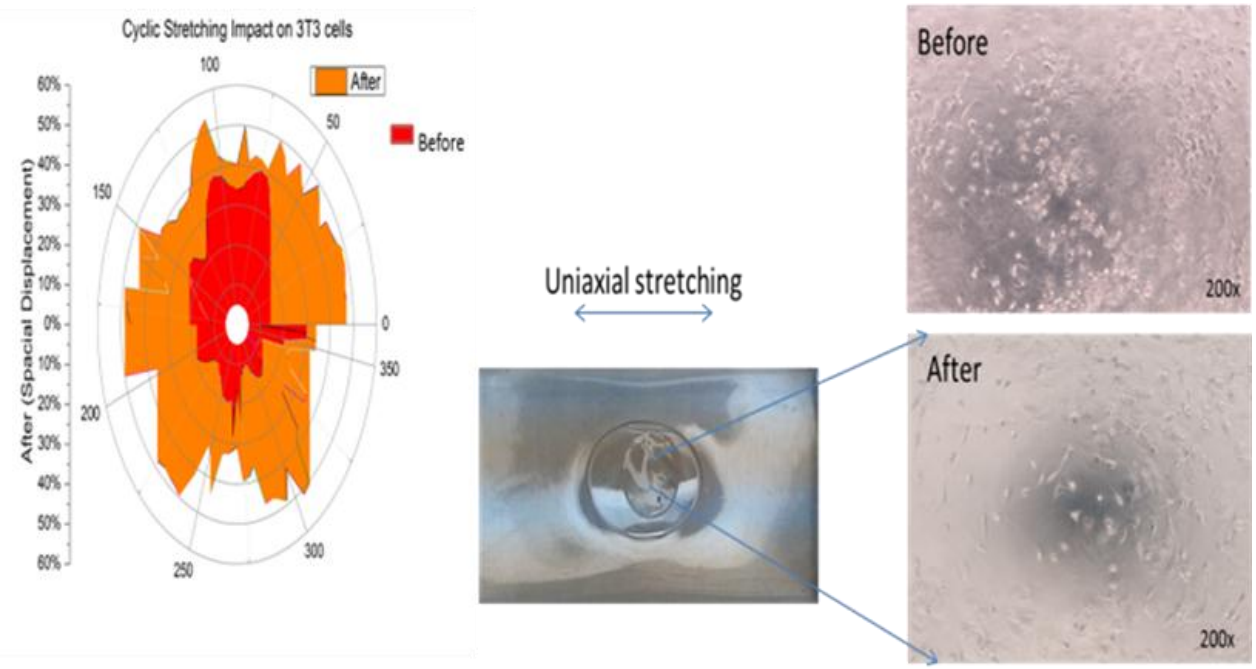


Figure 18 Maps of the before and after morphological changes of the cell population that was exposed to cyclic mechanical stretching over a period of a week.

It has been clearly observed that we had a significant spatial displacement of the cellular population from its original seeding area and more specifically the cells migrated perpendicularly from the stretching direction.

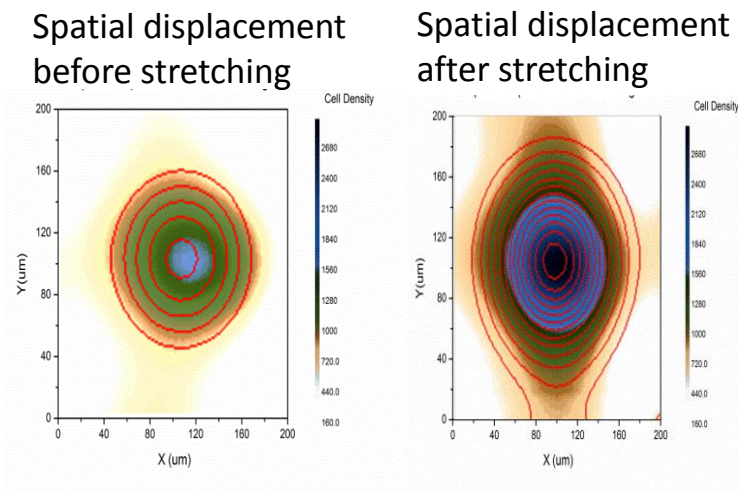


Figure 19 Computational simulation of the fibroblast population on top of the PDMS/hydrogel construct before and after stretching

To visualize the actin organization and nuclei of the fibroblasts adhered and proliferated on the hydrogel, we have stained the samples with fluorescently labeled phalloidin (Alexa Fluor® 488 phalloidin) and 4',6-diamidino-2-phenylindole (DAPI). Following, the samples were observed under confocal fluorescent microscope (DM6000 B, Leica).

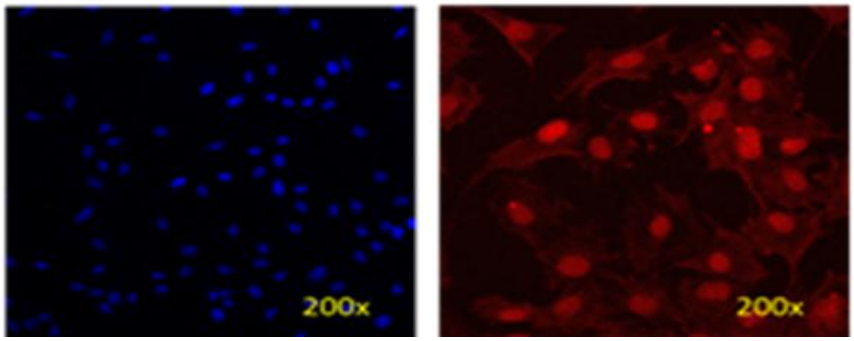


Figure 20 Fibroblasts stained with DAPI/ACTIN, grown on the hydrogel/PDMS and visualized before stretching.

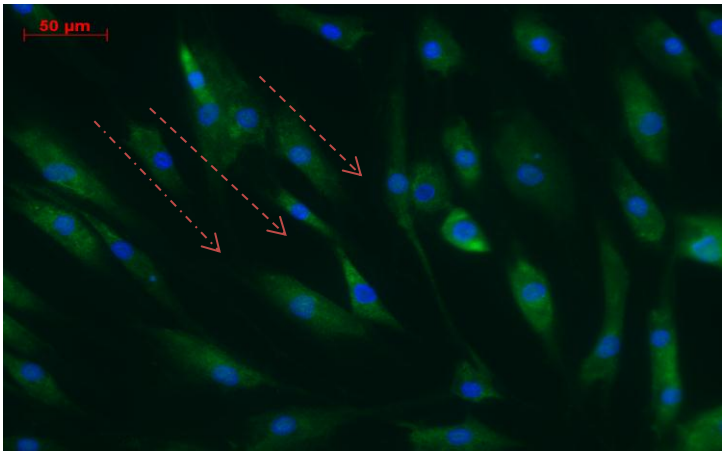


Figure 21 Fibroblasts seeded on top of the hydrogel/PDMS construct and exposed to cyclic mechanical stretching for 20min/3x/7days. Cells stained for DAPI/Actin

3.4 Conclusions

After studying the effect of cyclic mechanical stretching over a period of 7 days on a 2D cell culture, we conclude that there is a significant difference in cell orientation and alignment between the control group and the scaffold samples. We have examined 5 different images from each sample and have counted the number of cells proliferating in each image (0.2mm^2) by using ImageJ. We ran a T test to compare the number of cells on both groups and the results showed that stretching induced cell proliferation and doubling rates by 20% ($p<<5$).

It is remarkable that the cell clusters that were produced via the hanging drop method when the fibroblasts were seeded on the gels were significantly spread after stretching in a longitudinal orientation. More importantly, we have observed that over time the hydrogel substrate maintained its mechanical characteristics and its porous network has also been affected from the cyclic mechanical stretching with significant porous alignment that increased the matrix stiffness.

These results validated the performance of the mechanical stretching unit and demonstrated our capability to provide uniaxial cyclic stretching during cell culture with well-defined mechanical parameters (such as mechanical strain, strength, frequency and duty cycle) which can affect the cellular morphology and spreading.

Chapter 4: Effects of cyclic mechanical stretching on 3D cell culture and tissue morphogenesis

4.1 Introduction

The physical and mechanical properties of many engineered tissues can be enhanced through mechanical stimulation, although it is unclear what the most effective mechanical stimulatory cues are. As we have discussed in the introduction, artificial skin is impacted by mechanical stretching by having different proliferation rates, gene expression patterns, differentiation triggers etc. In the past, researchers have shown that 15 min of cyclic mechanical stretching can result to the upregulation of gene expression endothelial growth factor [77]. Our 3D construct that consists from a PDMS substrate and a softer hydrogel on top can achieve mechanical characteristics very closely to those of natural skin as we have shown in our previous study [78] .

To test our hypothesis, of developing aligned tissue with the aid of our 3D model, we have perfomed a 3D cell culture experiment and applied cyclic mechanical strain at a rate of 0.1 and frequency 1Hz for 2min, 3 times per day for a period of a week.

4.2 Methods

4.2.1 Cell proliferation and viability assay for 3D microenvironments

In order to test the cellular proliferation in the 3D construct we have used the MTS assay. The fibroblasts were exposed to the MTS tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (Promega Corporation, Madison WI) and the living cells were observable by the formation of a soluble formazan product. Following the culture was scanned for absorbance at 490nm and the amount of formazan that was calculated was directly proportional to the number of living cells.

In order to test the cell viability we have used the MTT assay that was slightly altered to meet the needs of 3D cell culture (prolonged incubation times). So the 3D gel constructs were exposed to MTT[3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide](ATCC, Manassas, VA). The viable cells were observed by the formation of a dark purple color which in turn led to the reduction of the MTT reagent in mitrochondria.

4.2.2 Statistical analysis

The cell culture proliferation rates were evaluated using an unpaired, two tailed Student's t-test in SPSS. Statistical differences were considered significant for $p<0.05$ and we used one-way analysis of variance (anova) to check the significance between more than two populations. With $p>0.1$.

4.3 Results

4.3.1 RGD-alginate synthesis for 3D cell culture in microporous scaffold

The culture model used in this experiment was developed through the conjugation of RGD peptide with our hydrogel system and was tested by using different RDG/alginate ratios and different stiffness. For our aligned microporous network we have conjugated 3.1×10^{8} RGD/mm^2 as shown in the reaction scheme below.

Figure 22 Conjugation of Arg-Gly-Asp (RGD) with alginate for enhanced cell adhesion in the microporous scaffold

The proliferation rate of the NIH3T3 fibroblasts in 3D were evaluated with normalized absorbance from an MTS assay, where 10kPa gels were statistically different.

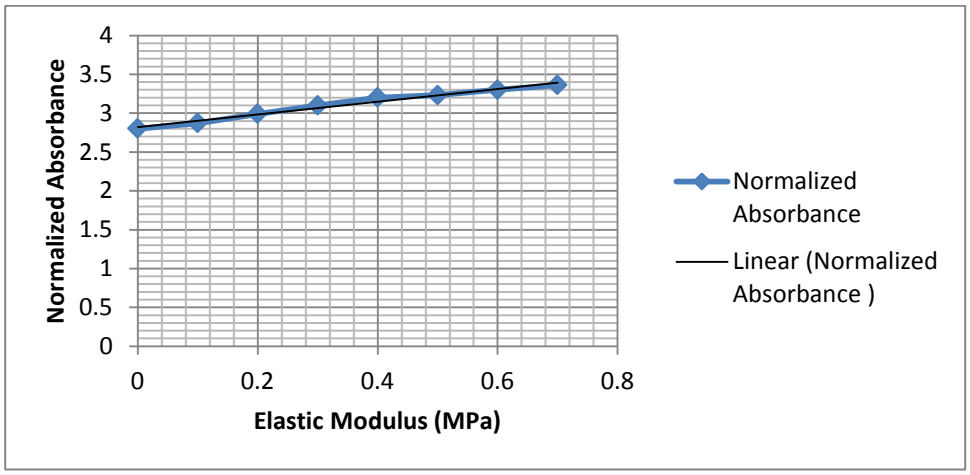


Figure 23 Effect of substrate stiffness on fibroblast proliferation

We have also varied the crosslinking ratios and measured the elastic moduli and strain with and without the microporous network in order to obtain a better understanding of the system. The degree of porosity has a significant effect on the mechanical properties, with the stiffness of the scaffold decreasing as porosity increases.

AAD/uronic acid	Modulus of hydro gel without micro pores(kPa)	Maximum strain of the gel without micropore (%)	Modulus of micro porous hydrogel (kPa)
0.0014	3	37.0 ± 4.2	8
0.0019	15	27.5 ± 3.8	17
0.0027	42	21.3 ± 2.5	51

Figure 24 Control of gel stiffness by changing the ratio of AAD crosslinker to uronic acids of the alginate

4.3.2 Engineered Skin approach with 3D cell culture

For this experiment, the scaffold was prepared under sterile conditions, the AAD-alginate was dissolved in sterile hood (AAD:EDC:NHS ratio was 0.05:0.1:0.05) and conjugate with RGD 3.1×10^8 RGD/mm^2 and filtered to remove impurities. Consequently, the gel disks of 10mm thickness were formed on top of the PDMS and they were washed 3x with sterile PBS and placed under UV for 30min. NIH3T3 fibroblasts were cultured as previously and seeded at a density of 10^6 cells/cm^2. The 3D cell culture was maintained for 7 days and the media was changed every second day.

We have applied cyclic mechanical stretching on the constructs on the 7^{th} day when they have reached 80-85% confluency . The samples were stretched at 0.1 strain, frequency of 1Hz for 20min, 3 times per day for 7 days.

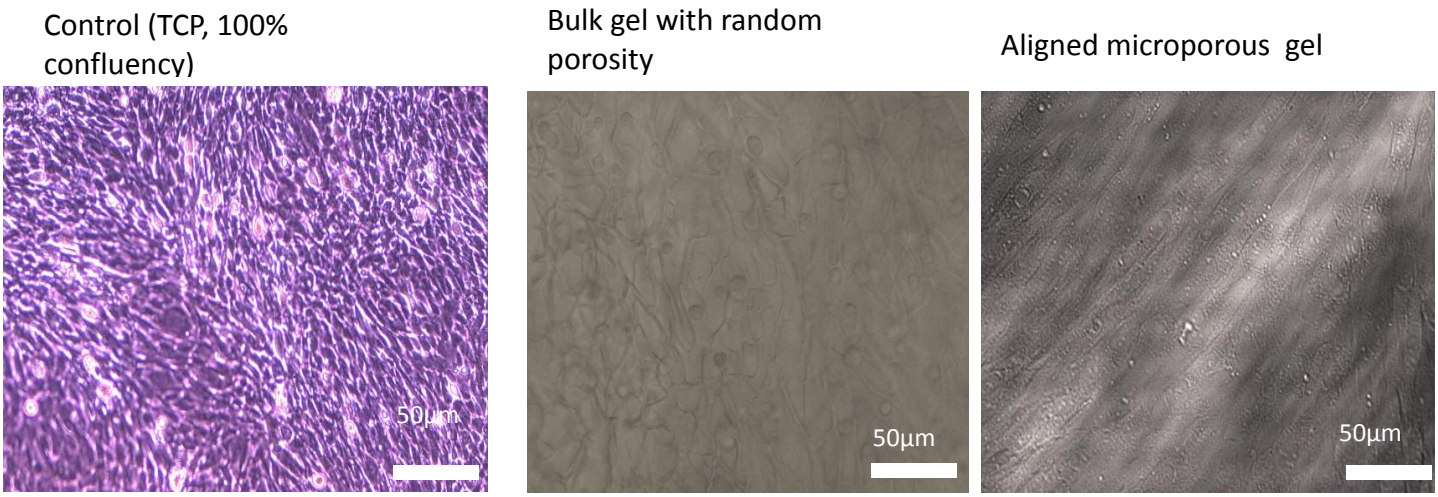


Figure 25 NIH3T3 fibroblasts cultured in TCP(left), in gel with random network orientation (center) and in aligned microporous gel(right)

Following the exposure to cyclic mechanical stretching, the fibroblasts were stained with DAPI to visualize their nucleus and with phalloidin for actin and were observed under the confocal microscope.

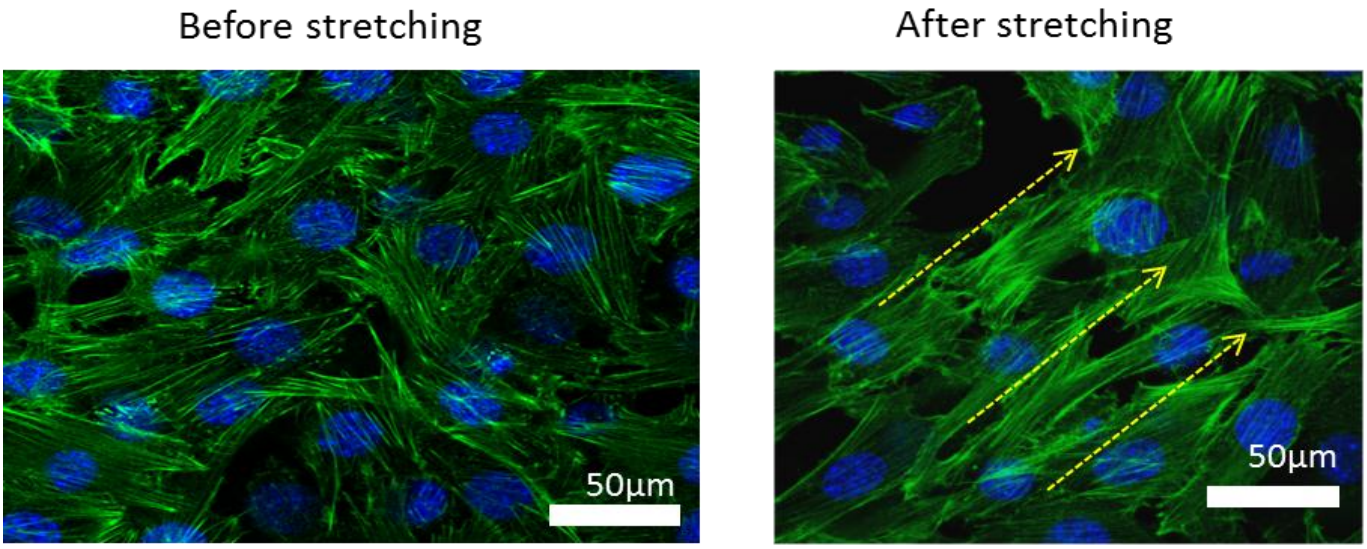


Figure 26 Confocal microscopy in 3D scaffolds after staining NIH3T3 cells with Alexa-488 phalloidin(green) and DAPI(blue) for actin and nucleus respectively.

Conclusions

Tissue engineering can provide clinical applications that will revolutionize conventional therapeutic pathways and give an end to severe clinical diseases. In combination with regenerative medicine it can alleviate the disparity between the donor organs available and the huge need for transplants. Billions of people are suffering from burn injuries, cuts and traumas worldwide, many of which cannot be treated with existing techniques such as autografts, due to their limited availability. Tissue morphogenesis can be achieved through a combination of tissue engineered tools although there are still many challenges to overcome regarding the efficient simulation of the human tissue properties. Engineered tissue remains a significantly weaker tissue and many techniques have been investigated in the past to enhance its elastic modulus. In our project we have showed that crosslinking modification in combination with mechanical stimulation can be a promising method to control the mechanical properties of tissues and eliminate biological hazards.

This study has provided a unique tool for many tissue engineering application that need to utilize the cyclic mechanical stretching and tune the matrix rigidity. Artificial skin is one primary application since this mechanically forces are naturally applied to it. Through experimentation, we found that matrix organization, and especially microporous alignment can play a major role in the mechanical properties of the tissue formed and cyclic mechanical stretching increases the stiffness of the substrate and the alignment of the cellular microenvironment.

Through these findings we can speculate that fibroblasts in real tissues like the epidermis of the skin are shielded from mechanical strain by the outer layer epidermis, similarly to the higher strain that our fibroblasts were enduring closer to the interface between the alginate and the PDMS substrate. A wide variety of mechanical stimulation profiles has been investigated and this allows us for better determination of the most effective stimulation profile necessary for improving the engineered tissue biomechanics. We have provided evidence that alterations in scaffold morphology can influence changes in cell spreading and are responsible for increases in tissue stiffness. In future studies, a detailed analysis of gene expression patterns in relation to strain is needed to pinpoint the root cause of the changes in the mechanical characteristics and in order to use this knowledge to improve artificial skin biomechanics.

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