An investigation into the effects of substrate properties on the mechanics of corneal epithelial cells

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An Investigation into the Effects of Substrate Properties on the Mechanics of Corneal Epithelial Cells

By

Preeti Holland

A thesis submitted to Loughborough University

For the degree of

Doctor of Philosophy

October 2018

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Abstract

Cells respond to mechanical changes in their extracellular environment, as reflected by various cell behaviours and observed through changes in the tissue biomechanics. Types of cell behaviour that are regulated by mechanical cues in the microenvironment of the cell are cell spreading, migration, proliferation and differentiation. Cell migration is a key part of many biological processes including corneal wound repair. Changes in the biomechanical properties of the cornea can be induced by refractive and therapeutic treatments and also by diseases of the eye or other illnesses.

A Rabbit Corneal Epithelial (RCE) cell line was used to study cell mechanics and cell migration. Polydimethylsiloxane (PDMS), a biocompatible silicone elastomer, was used as a substrate to culture RCE cells. In order to promote cell attachment and growth, the hydrophilicity of the PDMS surface was increased by treating it with oxygen-rich cold atmospheric pressure plasma, which was confirmed by surface characterisation techniques. Cell attachment and growth studies over time comparing plasma and non-plasma treated PDMS showed an increase in RCE cell growth and area coverage on plasma treated PDMS.

To investigate the effects of substrate stiffness on RCE cell behaviour, different PDMS samples were produced with differing Young’s modulus. Scratch wound migration assays were performed on RCE cells cultured on PDMS to see how differences in substrate stiffness affected the way that the RCE cells migrated. It was found that as the Young’s modulus of the PDMS sample decreased; the percentage wound closure of the RCE cells also decreased, which suggested that the softer PDMS samples had an effect on the cytoskeletal interactions between the cell and the substrate, stimulating the mechanosensing machinery of the RCE cells. Differences in cell attachment, motility, and morphology were also observed as the stiffness of PDMS decreased and it was thought that on the softest PDMS blends, mature focal adhesions had not formed. The differences in cell mechanics observed here were further studied at nanoscale levels using AFM. It was observed at the single cell scale, the general trend in Young’s modulus measured on different PDMS substrates was that as the Young’s modulus decreased, the stiffness of the nucleus, area next to the nucleus and the cell body also decreased in stiffness except on the cell edge. The results at the cell edge were more varied across the PDMS blends and the focal adhesions and actin stress fibres may have had an impact on the measurements obtained. Furthermore, the effects of mechanical stretching on PDMS–RCE cell samples were investigated using an in-house uniaxial stretching device. Optimisation and development is needed for the device to link the macroscale mechanical properties to the micro or nanoscale cell mechanics as observed through changes in cellular behaviours.
Acknowledgements

I would like to start by expressing my gratitude and thanks to my PhD supervisors Dr Yang Liu and Dr Pablo Ruiz. Their academic guidance and support over the years has been instrumental during my PhD. I would also like to thank the staff and students of the Centre for Biological Engineering and their support, friendship and guidance through my time studying at Loughborough.

In particular I would like to thank Matt, Emma, Maz and Jen for all the laughs, hugs and copious tea breaks! Without them my time at Loughborough and beyond just wouldn’t be the same, thank you for you for your continual support and friendship. I would also like to thank Abby Wilson for her contribution to my PhD work and for listening to my rants about work.

Thank you to the plasma group at Loughborough for letting me use their equipment and also for making me feel part of the group and welcome in their labs. Special thanks to Alex Shaw and Dr Felipe Iza for their guidance and support.

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A big thank you goes to Jo for always being there no matter what and I am so appreciative of your support and friendship through the PhD and in life. I would also like to thank Swati, Hannah, Lizzie, Ali and Meera for always motivating me and believing in me and of course for being amazing friends.

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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASTM</td>
<td>American society for testing materials</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSC</td>
<td>Biological safety cabinet</td>
</tr>
<tr>
<td>CAP</td>
<td>Cold atmospheric pressure</td>
</tr>
<tr>
<td>CH</td>
<td>Corneal hysteresis</td>
</tr>
<tr>
<td>CMFDA</td>
<td>5-chloromethylfluorescein diacetate</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRF</td>
<td>Corneal resistance factor</td>
</tr>
<tr>
<td>CS/DS</td>
<td>Chondroitin/dermatan sulfate</td>
</tr>
<tr>
<td>CTF</td>
<td>Cell traction force</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6 – Diamidino – 2 – Phenylindole, Dihydrochloride</td>
</tr>
<tr>
<td>DMEM: F12</td>
<td>Dulbecco’s modified eagle medium: Ham’s F12 medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ECACC</td>
<td>European collection of authenticated cell cultures</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ESPI</td>
<td>Electronic speckle pattern interferometry</td>
</tr>
<tr>
<td>EthD - 1</td>
<td>Ethidium homodimer – 1</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically modified organism</td>
</tr>
<tr>
<td>GPa</td>
<td>Gigapascal</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanidine exchange factor</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
</tr>
<tr>
<td>KPa</td>
<td>Kilopascals</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>LASIK</td>
<td>Laser assisted in situ keratomileusis</td>
</tr>
<tr>
<td>LECs</td>
<td>Limbal epithelial crypts</td>
</tr>
<tr>
<td>LESCs</td>
<td>Limbal epithelial stem cells</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascals</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>ORA</td>
<td>Ocular response analyser</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascals</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PET</td>
<td>Poly (ethylene terephthalate)</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>pHHEMA</td>
<td>Poly (2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>PRK</td>
<td>Photorefractive keratectomy</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly (vinyl alcohol)</td>
</tr>
<tr>
<td>QI mode</td>
<td>Quantitative imaging mode</td>
</tr>
<tr>
<td>Ra</td>
<td>Average roughness</td>
</tr>
<tr>
<td>RCE</td>
<td>Rabbit corneal epithelial</td>
</tr>
<tr>
<td>RK</td>
<td>Radial keratotomy</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>Rq</td>
<td>Root mean squared average of the height profile</td>
</tr>
<tr>
<td>Rt</td>
<td>Peak to valley roughness</td>
</tr>
<tr>
<td>SCCM</td>
<td>Standard cubic centimetres per minute</td>
</tr>
<tr>
<td>SLM</td>
<td>Standard litres per minute</td>
</tr>
<tr>
<td>SLS</td>
<td>Scientific laboratory supplies</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>TACs</td>
<td>Transit amplifying cells</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td>TEVGs</td>
<td>Tissue engineered vascular grafts</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>UTS</td>
<td>Ultimate tensile strength</td>
</tr>
<tr>
<td>UV</td>
<td>Water contact angle</td>
</tr>
<tr>
<td>WCA</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>XPS</td>
<td>Three-dimensional</td>
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Chapter 1

Introduction and Literature Review
1.1. Introduction

Corneal biomechanics can be affected by several factors including injury, surgery and by illness or disease. Change in the biomechanical properties can impact different cellular processes such as cell adhesion, proliferation and cell migration (Kling and Hafezi, 2017). Cell migration is a key part of a number of physiological processes such as the immune response, cancer cell invasion and also corneal wound healing (Doyle et al., 2013). Changes in the mechanical properties of the cell microenvironment can cause differences in cell adhesion and the formation of focal adhesions between the cell and extracellular matrix (ECM) can be affected, leading to differences in cell motility (Moreo, García-Aznar and Doblaré, 2008).

The interactions between the cell and the ECM or substrate are critical for understanding the effects of biomechanical changes in the cornea on cell function and how this could impact corneal wound healing (Dupps and Wilson, 2006; Kurniawan, Chaudhuri and Lim, 2016). The single cell and collective cell mechanics are important in developing tissue constructs for therapies or understanding key parameters affecting the mechanics at a cellular level, leading to a better knowledge of the cellular scale mechanical properties as well as the cornea as a whole.

In the review of the literature, the current knowledge of the structure and function of the cornea is reviewed in terms of its biomechanical properties. The cellular processes affected by mechanical properties are also discussed, as well as methods used to investigate the biomechanical properties of single cells and the cell population.
1.2. The Structure and Function of the Cornea

The cornea is the primary refractive element of the eye and contains one of the most highly organised extra-cellular matrices (ECM) in the human body. The cornea is the tissue that forms the front surface of the eye and it is optically clear. This transparency is maintained by the highly organised collagen fibril bundles in the stroma of the cornea, called the lamellae (Kim et al. 2012).

The human cornea is a complex structure and is composed of several layers of different cell types. From the anterior to the posterior part of the cornea, the six layers of the cornea are; the epithelial layer with basement membrane, Bowman’s layer, stroma, Dua’s layer, Descemet’s membrane and endothelial layer (Daniels et al., 2001; Dua et al., 2013). A schematic cross section of the structure and layout of the cornea is shown in Figure 1.

![Diagram of the human cornea](image)

Figure 1 - A representative diagram of the human cornea highlighting the layers through a cross section. (Drawing adapted from (Daniels et al. 2001; Dua et al. 2013)).

Each layer plays an important role in the function of the cornea and the eye as a whole. The corneal epithelium consists of non-keratinised, stratified squamous epithelial cells. It is connected to the peripheral limbal epithelium and the conjunctival epithelium and they collectively cover the ocular surface (Ebrahimi, Taghi-Abadi and Baharvand, 2009), as depicted in Figure 2.
The corneal epithelium functions as a barrier, protecting the eye from pathogens and also from fluid loss. The tight junctions between neighbouring epithelial cells in the corneal epithelium produce this protective barrier on the outer surface of the eye (Lu, Reinach and Kao, 2001). The Bowman’s membrane is an acellular layer that is located anterior to the stroma and just below the basement membrane underlying the epithelium. It consists of collagen fibrils organised randomly within an extra-cellular matrix (ECM) (Lagali, Germundsson and Fagerholm, 2009).

The stroma accounts for about 90% of the mass of the cornea and the spacing and alignment of these collagen fibrils is crucial for corneal transparency and structure (Levis and Daniels, 2009). The corneal stroma is avascular and plays a key role in the function of the cornea in terms of protection, and transmittance and refraction of light (Ebrahimi, Taghi-Abadi and Baharvand, 2009). Keratocytes are the main cell type of the stroma, which also consists of proteoglycans and glycosaminoglycans. Keratocytes are also known as the corneal fibroblasts and are mesenchymal-derived cells of the adult cornea (West-Mays and Dwivedi, 2006). These keratocytes are usually in a quiescent state in vivo (Lakshman, Kim and Petroll, 2010). They are sparsely populated within the stroma but are able to create a network of cellular connections through dendritic processes (Lakshman, Kim and Petroll, 2010). The majority of the stroma is composed of water and collagen with the keratocytes distributed through the structure (Daniels et al., 2001).

The corneal stroma is able to maintain its structure and function due to the highly organised ECM (Dupps and Wilson, 2006). The specific orientation and spacing of collagen fibres and other matrix molecules that are present in the corneal stroma are thought to be critical for maintaining the transparency of the stroma and therefore the cornea (Pajoohesh-Ganji and Stepp, 2005). The stroma
in weight is made up of approximately 78 % water, 15 % collagen, 7 % non-collagenous proteins, proteoglycans and salts (Dupps and Wilson, 2006). Between the posterior stroma and Descemet’s membrane, recently a novel, acellular layer has been identified and named Dua’s layer. It is a well-defined, strong layer and its discovery will have a major impact on posterior corneal surgery, understanding of certain diseases affecting the posterior cornea and corneal biomechanics (Dua et al., 2013; Zaki et al., 2015).

Underneath the corneal stroma and Dua’s layer is the Descemet’s membrane, which is another basement membrane of the corneal endothelium. The function of the endothelium is to maintain the corneal stroma in a relatively dehydrated state and the presence of focal tight junctions prevents fluid flow into the stroma (Ebrahimi, Taghi-Abadi and Baharvand, 2009). The endothelial layer causes net fluid transport out of the stroma into the anterior chamber. This prevents stromal swelling which in turn maintains corneal clarity. The endothelial fluid transport activity and epithelial cell renewal help to maintain stromal thinness and optical refraction (Lu, Reinach and Kao, 2001). Nutrients are pumped into the stroma by the endothelial cells and they also pump excess water out of the cornea. These nutrients will then diffuse throughout the cornea supplying the stromal fibroblasts or keratocytes and corneal epithelium. The endothelial cells help to maintain the quality of the corneal stroma and therefore they have a critical role in maintaining transparency (Pajoohesh-Ganji and Stepp, 2005). The lacrimal gland is an exocrine gland and contributes to several components of the tear film that is made up of three layers; an inner mucin coating, an aqueous component in the middle and a lipid overlay (Conrady, Joos and Patel, 2016). The tear film prevents the invasion of pathogens through the ocular surface and lubricates the eye coating the anterior surface. It also enables gas exchange and provides the important nutrients required to keep the cornea transparent and avascular. There is a clear blood-tear barrier, creating a compositional difference between tear fluid and blood (Farandos et al., 2015; Conrady, Joos and Patel, 2016).

1.2.1. Limbal stem cells and corneal epithelial stem cells

The limbus is the region between the corneal epithelium and the conjunctival epithelium (Figure 2) and it is at this junction that limbal epithelial stem cells (LESCs) reside. The stem cells are located in the basal layer of the limbus, at the vascularised junction between the two different epithelia (Notara et al., 2010). In 1971, it was first suggested that the corneal epithelium is maintained and renewed by a population of cells located at the limbus (Davanger and Evensen, 1971). The X, Y, Z hypothesis of corneal epithelial maintenance was then proposed in 1983. This hypothesis stated that when corneal epithelial cells are shed from the surface of the corneal epithelium (Z component), the cells are replaced by migration of cells from the periphery of the corneal epithelium to the centre (X)
and migration of cells from the basal layer of the corneal epithelium to the surface (Y) (Thoft and Friend, 1983). The homeostasis of the cornea is therefore \( X + Y = Z \) and if \( X \) or \( Y \) do not occur and fail to replace the lost cells, this causes problems that occur in limbal stem cell deficiency (LSCD) (Ahmad et al., 2010).

Following this research, there has been further evidence supporting the presence of stem cells at the limbus. Cotsarelis et al showed that the limbal epithelial basal cells were slow-cycling, which is a characteristic of a quiescent stem cell (Cotsarelis et al., 1989). This was demonstrated by using tritiated thymidine, which the cells retained for longer periods, proving that they were slow-cycling and not highly proliferative (Dua and Azuara-Blanco, 2000). It was also shown that the limbal basal cells have greater proliferative capacity when cultured in vitro compared to central and paracentral corneal epithelial cells (Pellegrini et al., 1999). More evidence supporting the presence of stem cells in the limbus showed that damage or surgical removal of the limbus caused reduced healing by non-corneal epithelium (Chen & Tseng 1991; Huang & Tseng 1991).

This evidence suggested that there were a population of stem cells present in the limbus and that they resided in a specific location due to their surrounding microenvironment. This microenvironment is created within the stem cell niche and the LESC\( ^{s} \) are maintained in an undifferentiated state until stimulated by mechanical or biochemical factors (Notara and Daniels, 2008). The LESC niche within the limbus is thought to be provided by the Palisades of Vogt, which are papillae-like structures that supply the LESC\( ^{s} \) with a physical protective environment, away from the harsher external environment. It has been observed that the stem cells lie between the palisades at the base of the epithelial papillae (Schlötzer-Schrebradt and Kruse, 2005). LESC\( ^{s} \) are also protected from shear forces due to the junction between the stroma and the limbal epithelium. The close proximity of the underlying limbal blood vessels to the LESC\( ^{s} \) ensures that they receive enough nutrients (Boulton and Albon, 2004).

Understanding the stem cell niche is a fundamental step towards the use of stem cell therapies for regeneration and repair of tissues. Further investigations into the micro-anatomy of the limbus identified novel anatomical structures that extend outwards from the Palisades of Vogt, named the limbal epithelial crypts (LECs) and these structures showed characteristics of a stem cell niche. Cells that were found within the LEC\( \text{s} \) also stained positive for corneal epithelial marker cytokeratin 14 and a stem cell marker ABCG2 transporter protein (Dua et al., 2005). Later studies found that there were two LESC niche-like structures, the limbal crypts (LC) and the focal stromal projections (FSP) and these structures were present alongside the Palisades of Vogt (Shortt et al., 2007). It was observed that there was an uneven distribution of these structures around the circumference of the
cornea and the LESC\textsubscript{s} seemed to congregate in the inferior and superior regions of the human limbus (Notara and Daniels, 2008).

In terms of identification and characterisation of LESC\textsubscript{s}, there is currently no single stem cell marker that specifically identifies the limbal epithelial stem cell (Notara et al., 2010). Therefore instead of using a single marker, the presence of stem cell associated markers and the lack of differentiation markers can be used to identify putative LESC\textsubscript{s}. Table 1 highlights the stem cell markers used to identify the LESC\textsubscript{s} and these markers can be used along with the absence of differentiation markers such as cytokeratin 3/12 and connexion 43. However, many of these markers also stain transit amplifying cells (TAC\textsubscript{s}), which are fast-dividing progenitor cells and not purely stem cells. It is therefore necessary to look at both stem cell markers and morphology of the LESC\textsubscript{s} when identifying and characterising the LESC\textsubscript{s} (Schlötzer-Schrehardt and Kruse, 2005).

<table>
<thead>
<tr>
<th>Stem Cell Marker</th>
<th>Type of Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63, alpha isoform (\Delta Np63\alpha)</td>
<td>Transcription factor involved in morphogenesis</td>
</tr>
<tr>
<td>ABCG2</td>
<td>ATP-binding cassette transporter protein</td>
</tr>
<tr>
<td>Notch 1</td>
<td>Ligand activated transmembrane receptor</td>
</tr>
<tr>
<td>C/EBP(\delta)</td>
<td>CCAAT enhancer binding protein delta, transcription factor</td>
</tr>
<tr>
<td>Bmi1</td>
<td>Polycomb group repressor</td>
</tr>
<tr>
<td>Cytokeratin 14, 15, 19</td>
<td>Intermediate filament protein family, part of cytoskeleton</td>
</tr>
</tbody>
</table>

Although the work discussed in Chapters 2-7 uses rabbit corneal epithelial (RCE) cells, a more robust cell line, further work could be carried out to investigate LESC\textsubscript{s} following the optimisation of processes that are used in this research (See Chapter 7 for future work Section). The effect of changes in mechanical properties in the extracellular environment on the LESC\textsubscript{s} could allow further investigation into the stem cell niche, the response of LESC\textsubscript{s} to mechanical stimulation and the impact on LESC migration and corneal wound healing.
1.2.2. Factors affecting the biomechanics of the cornea

1.2.2.1. Corneal wound healing and the processes involved

The corneal epithelium or the outermost layer of the cornea is continually renewed and this process is critical in maintaining its function. If the surface of the cornea is wounded, the tight junction integrity of the epithelial layer may be compromised. This could lead to a collapse in cell membrane permeability and its barrier function and will only be restored when the damaged surface is renewed by differentiated corneal epithelial cells. Wounding of the epithelial layer could lead to loss of corneal transparency due the effect on fluid transport, which could increase stromal hydration (Lu, Reinach and Kao, 2001). Renewal of the corneal epithelium is maintained by the limbal epithelial stem cells (LESCs) that reside in the limbus and proliferate and differentiate to replace the terminally differentiated corneal epithelial cells. It has been shown that corneal epithelial regeneration occurs by a centripetal migration of limbal epithelial stem cells from the limbus. This is characteristic of the transit amplifying cells that are migrating towards the central cornea and upwards to the surface of the cornea, becoming terminally differentiated corneal epithelial cells (Lu, Reinach and Kao, 2001; Dua et al., 2005), as shown in Figure 3.

![Diagram showing limbal epithelial stem cells](image)

Figure 3 – Representative diagram showing the location of the limbal epithelial stem cells (LESCs) found within a limbal stem cell niche (Palisades of Vogt) that produce transit amplifying cells and shows their migration to the central cornea as terminally differentiated cells.
Following an injury to the corneal epithelium, cytokines are released from the injured epithelium, which include interleukin (IL) – 1 and tumour necrosis factor (TNF) alpha (Wilson, Liu and Mohan, 1999), epidermal growth factor (EGF), bone morphogenetic proteins (BMP) 2 and 4 and platelet derived growth factor (PDGF) (Tuominen et al., 2001). The underlying stromal keratocytes are then stimulated to respond due to the presence of these factors along with others derived from the tears. Synthesis of Fas ligand by an IL-1 mediated synthesis in the keratocytes occurs and they bind to Fas receptors on nearby keratocytes to induce apoptosis, or programmed cell death (Wilson, Liu and Mohan, 1999). Following initial keratocyte apoptosis, growing numbers of cells then undergo the process of necrosis, which is a pro-inflammatory process (Wilson et al., 2001).

Once the epithelial barrier had been compromised by injury, the effects of the epithelial and lacrimal cytokines were potentiated due to the increased access to the stromal layer of the cornea (Dupps and Wilson, 2006). The remaining keratocytes begin to proliferate and migrate 12 to 24 hours after injury, which in turn produces activated keratocytes, fibroblasts and potentially myofibroblasts that re-populate the damaged stroma (Fini, 1999). Pro-inflammatory chemokines from the corneal epithelium or stromal keratocytes trigger infiltration of T cells, macrophages or monocytes and polymorphonuclear cells into the stroma in the first 24 hours after injury, in response to IL-1 and TNF-alpha (Dupps and Wilson, 2006). The limbal blood supply allows these cells to reach the injured area as well as from the tear film and they are involved in the phagocytosis of apoptotic and necrotic cell debris (Helena et al., 1998; Wilson, Chaurasia and Medeiros, 2007).

Between one and two weeks after injury, myofibroblasts can be visualised in the anterior stroma below areas of epithelial basement membrane disturbance (Netto et al., 2006). These cells are a critical part of the wound healing process and are thought to be derived from keratocytes that have responded to transforming growth factor (TGF)-beta and are identified through their contractile pseudopodia (Jester and Ho-Chang, 2003). Myofibroblasts also have a role in corneal haze formation following injury and during stromal remodelling. This is because they have reduced transparency, meaning that the fine balance between the regeneration of the stroma and fibrosis is largely controlled by these cells, impacting on corneal transparency (Stramer, 2003).

If the epithelial basement membrane has been injured, a fibronectin scaffold is deposited due to the presence of EGF and this supports epithelial repair on its surface (Suzuki et al., 2003). The re-epithelialisation of the cornea following a wider defect such as the type used in photorefractive keratectomy (PRK) typically occurs in three to five days after the insult. Over several weeks the myofibroblasts disappear but the process may carry on for months or years (Helena et al., 1998; Netto et al., 2006). In terms of corneal wound healing following laser assisted in situ keratomileusis
LASIK) or PRK, the magnitude of the attempted treatment is clearly linked to the strength of the corneal response (Dupps and Wilson, 2006).

1.2.2.2. The effects of injury, surgery and illness on corneal biomechanics

The majority of the response to corneal injury is dominated by the stroma as it is the largest layer of the cornea, representing the largest proportion of the defining biomechanical properties of the cornea (Dupps and Wilson, 2006). Each layer within the cornea has a different density and orientation of collagen and therefore the contribution of each layer to the overall biomechanical resistance of the cornea varies. The layers of the cornea that contain collagen fibrils, the Bowman’s layer and the stroma, provide most of the tensile strength (Meek and Boote, 2009).

Within the Bowman’s membrane, the collagen lamellae are densely packed in providing corneal stability, in particular following laser ablative surgery (Kling and Hafezi, 2017). The corneal epithelium has an insignificant role in the tensile strength of the overall cornea. The epithelial and endothelial layers of the cornea are only cellular layers and have no direct contribution to the stiffness of the cornea (Elsheikh, Alhasso and Rama, 2008a). However these layers may have an indirect role in the overall corneal stiffness by being able to control the hydration of the cornea (Kling and Hafezi, 2017). Dua’s layer, a recently discovered layer found between the stroma and Descemet’s membrane, has been suggested to have a significant contribution to the mechanical properties of the cornea due to its mechanical strength. However further investigations are required into its true role in the overall stiffness of the cornea (Dua et al., 2013; Kling and Hafezi, 2017). The low stiffness of the Descemet’s membrane enabled it’s tolerance to a broad range of intraocular pressures (IOP) and may also help to prevent the mechanical stress in the stroma spreading to the endothelium (Dupps and Wilson, 2006; Thomasy et al., 2014).

Corneal keratocytes, which are the cells that reside in the stroma, also have a key role in wound repair. Following injury they can be stimulated to undergo cell death, to change phenotype to induce regeneration and repair of the tissue, or stimulate scar formation (West-Mays and Dwivedi, 2006). The cornea is directly exposed to environmental factors and is therefore susceptible to damage or injury due to physical or chemical factors (Kim et al. 2012). Damage to the cornea can also occur following corrective refractive surgery such as excimer laser PRK and LASIK which uses photoablation to change the shape of the cornea for the desired refractive power (Dupps and Wilson, 2006). This causes keratocyte death and in some patients, normal tissue healing occurs leading to scar formation and fibrosis of the tissue. Ultimately this can lead to permanently reduced clarity of the cornea (Kim et al. 2012).
Myofibroblasts are fibroblastic cells whose physiological and ultrastructural properties are like smooth muscle cells (Jester et al., 1999). Corneal myofibroblasts play a role in repairing the cornea following a penetrating injury and they are well suited to this role due to their ability to contract wounds, produce and secrete ECM and form adhesions with the surrounding substrate (Wilson, 2012). This cell type are not normally detected in unwounded corneas (Chaurasia et al., 2009). The generation of myofibroblasts and their contraction are therefore thought to benefit the processes that restore the integrity of the eye following traumatic and surgical lacerations of the cornea (Wilson, 2012). They also aid with the strength of the wound between donor tissue and the recipient after penetrating keratoplasty and are also present in the flap edge that is formed in LASIK (Netto et al., 2007). However after other surgeries, the presence of myofibroblasts is thought to be stimulated as a response to injury. For example, after PRK, the patient can develop clinically significant late haze where the stroma becomes opaque. This occurs one to three months after surgery and can lead to continuous corneal opacity, deterioration of the effects of the surgery and the development of irregular astigmatism (Mohan et al., 2003). Mild haze does normally occur in the first few weeks to months following PRK, including in corneas with perfect clinical outcomes (Wilson, 2012). In all tissues where myofibroblasts are present, high levels of ECM including collagen type 1 and fibronectin are excreted. This may have a part in the regeneration of tissue but they also contribute to corneal opacity even after myofibroblasts have gone from the wound site. In order to maintain corneal transparency, the additional ECM produced must be reabsorbed by keratocytes and other cells present if possible (Karamichos et al., 2010).

Following surgeries such as radial keratotomy (RK) that produce an incisional wound in the cornea, myofibroblasts are formed within the wound. The incisions cause local weakness in the cornea therefore modifying its biomechanical properties and overall structure (Petroll, Cavanagh and Jester, 1998). The intracellular stress fibres of the myofibroblasts aligned parallel to the long axis of the incisional wound to enable contraction and wound closure, altering the biomechanical properties in response to injury (Petroll, Cavanagh and Jester, 1998; A. Kim et al., 2012). Incisional surgeries were replaced by laser techniques such as PRK and LASIK but the fundamental principle of refractive surgery was that the incision or excision of tissue can produce defined changes in the corneal curvature hence changing the refractive properties (Jester, Petroll and Cavanagh, 1999). This could cause changes to the biomechanical properties of the cornea due to structural changes and could affect the success of the surgical procedure on vision improvement. Following surface ablation surgeries like PRK, myofibroblasts tend to be found in the anterior stroma beneath the epithelial basement membrane and are thought to be part of the wound healing response here (Mohan et al., 2003).
The biomechanical properties of the cornea can also be affected by certain systemic diseases. It has been found that patients with Diabetes mellitus have an increased corneal resistance. However, diabetes also creates a protective effect on the onset and severity of diseases such as keratoconus (Kuo et al., 2006; Snibson, 2010; Kling and Hafezi, 2017). Keratoconus is a bilateral ectatic disease that causes the thinning of the cornea due to a loss of collagen fibril orientation and a change in shape of the cornea to a more conical shape due to the outward protrusion of the cornea. This leads to biomechanical weakening and reduced visual acuity and astigmatism (Li et al., 2008; Snibson, 2010). Other conditions such as Ehler-Danlos syndrome and osteogenesis imperfecta have been reported to have a susceptibility to keratoconus due to both these conditions directly affecting the synthesis of collagen. There is an increased potential for corneal ectasia to develop in Ehler-Danlos and osteogenesis imperfecta patients due to the lack of a stable collagen network (Kenney and Brown, 2003; Kling and Hafezi, 2017).

Characterising the biomechanical properties of the cornea in vivo is challenging and invasive techniques have been used for this purpose such as injecting a saline solution into the anterior chamber or imaging the cornea by indentation of the central region. This led to the development of a non-invasive in vivo technique called the Ocular Response Analyser (ORA) to characterise the biomechanical properties of the cornea (Piñero et al., 2010). The commercially available ORA (Reichert Inc) uses a high-speed air puff technique and from the force required to flatten a constant area of the cornea, can quantify the dynamics of corneal deformation and recovery. This allows two biomechanical parameters to be measured; corneal hysteresis (CH) and corneal resistance factor (CRF) (Dupps and Wilson, 2006; Pepose et al., 2007; Shah et al., 2007). Several studies have been carried out using the ORA for non-invasive testing of the changes in corneal biomechanics following surgical procedures and in some pathological processes. In keratoconus, a reduction in ORA parameters measured, CH and CRF were observed (Piñero et al., 2010). The biomechanical changes observed in keratoconic corneas were found to be a result of the changes in the collagen lamellar structure and distortion of the orthogonal matrix that occurs in this condition, leading to the thinning of the central cornea and changes in corneal curvature (Meek et al., 2005).

Particular components of the ECM such as GAGs and proteoglycans (PGs) have a key role in the arrangement of the ECM and its transparency within the cornea. PGs consist of a core protein with one or more GAG side chains covalently attached. PGs are divided into three major groups; those that are involved in plasma membranes, can bind to hyaluronan or modify collagen fibril formation (Hassell and Birk, 2010). The different types of GAG side chains are chondroitin/dermatan sulfate (CS/DS), keratan sulfate (KS) and heparan sulfate (HS) (Hassell and Birk, 2010). There is a strong
interaction between PGs and other components of the ECM in the cornea and it is thought that keratan sulfate PGs have a role in regulating collagen fibril diameter and dermatan sulfate PGs can govern the spacing between collagen fibrils in the corneal stroma and contribute to the lamellar adhesion properties of the collagen in the cornea (Michelacci, 2003). In ectatic disorders of the cornea, such as keratoconus or macular corneal dystrophy, it was found that the amount of highly sulfated keratan sulfate PGs were reduced or absent and this could have an impact on the biomechanical changes that occur in these types of conditions (Funderburgh et al., 1990; Ihanamäki, Pelliniemi and Vuorio, 2004).

1.2.2.3. Mechanical properties of the cornea

The stroma consists of lamellae, which are made up of bundles of collagen fibrils (A. Kim et al., 2012). Interweaving of collagen bundles between neighbouring lamellae provides a fundamental physical base for shear resistance and the transfer of loads between lamellae (Dupps and Wilson, 2006). When the anterior basement membrane, anterior stroma and Descemet’s membrane were compared between human and rabbit corneas, the structures of the human cornea were evidently stiffer in comparison to the rabbit cornea (Last et al., 2012). The collagen organisation was found to be significantly different between rabbit and human corneas and was thought to be contributing factor to the observed differences in the biomechanics of rabbit and human stroma (Thomasy et al., 2014). The collagen architecture observed in the human stroma was found to show greater collagen fibre interweaving in the anterior 80% of the stroma and a parallel arrangement of collagen fibres only in the posterior 20% of the stroma. When compared to the rabbit stroma, there was found to be a greater proportion of parallel collagen fibres in the rabbit stroma and differences in the spacing and distribution of keratocytes (Thomasy et al., 2014). The increased collagen intertwining and crosslinking within the anterior stroma were suggested to cause the higher elastic modulus that was observed in the human cornea (Winkler et al., 2011).

Most biological tissues including the cornea exhibit viscoelastic properties in that they have both elastic and viscous material characteristics. The cornea in terms of material science is a complex anisotropic composite and there are two main properties that can be identified in corneal tissue due to its viscoelastic nature: elasticity or static resistance and damping or viscous resistance (Dupps and Wilson, 2006; Piñero et al., 2010). Viscoelastic materials have a dynamic deformation response to force which is time-dependent. The elastic behaviour refers to the reversible deformation of a material under stress, as shown by the slope of a stress-strain curve. In viscoelastic deformation, hysteresis is observed in the stress-strain curve of a viscoelastic material between the loading and unloading cycle (Figure 4), which shows the energy lost as heat during viscous deformation (Wang, Tian and Zheng, 2016; Kling and Hafezi, 2017). This is known as hysteresis and this behaviour is a
characteristic property of biological tissues and the loss of energy in this process is linked to viscosity. This defines the difference in force-deformation responses of materials under loading and unloading cycles (Wang, Tian and Zheng, 2016).

In corneal tissue, the elastic properties of the cornea mainly arise from the collagen fibres and their response to mechanical loading. Viscous properties of the corneal tissue are caused by water diffusion and electrostatic interactions between collagen and glycosaminoglycans (GAGs) (Kling and Hafezi, 2017). When a load is applied to an elastic material without pre-loading, initially the collagen fibrils are crimped resulting in a slow uptake of the load which can be seen on the example of a stress-strain curve show in Figure 4, also showing typical stress-strain curve for viscoelastic materials.

![Diagram showing the representative stress-strain curves for elastic and viscoelastic material.](image)

**Figure 4** – Diagram showing the representative stress-strain curves for elastic and viscoelastic material. Hysteresis is observed between the loading and unloading cycle and the area represents the energy lost during the viscous deformation as heat.

This is followed by elastic deformation as the collagen fibres straighten, which ultimately leads to strain-stiffening as the maximum fibril load is approached. When a higher load is exerted past the elastic region, permanent plastic material behaviour can be observed as the deformation of the material is irreversible under the applied load and eventually will lead to failure of the material (Dupps and Wilson, 2006; Kling and Hafezi, 2017). The elasticity of a material is measured using Young’s modulus and is defined as the slope of the tangent in the stress-strain curve. The equation for Young’s modulus is shown below and is derived from Hooke’s law which describes the linear relationship between stress and strain in relatively small material deformations:

\[
\text{Stress} \quad \text{Elastic deformation} \quad \text{Plastic deformation} \quad \text{Strain}
\]

\[
\text{Stress} \quad \text{Elastic deformation} \quad \text{Strain}
\]

\[
\text{Elastic material} \quad \text{Viscoelastic material}
\]

\[
\text{Elastic deformation} \quad \text{Strain}
\]

\[
\text{Stress} \quad \text{Hysteresis}
\]
\[ E = \frac{\sigma}{\varepsilon} \]

Equation 1 – Young’s modulus (Beer, 2012). Where \( E \) is Young’s modulus, \( \sigma \) is the engineering stress and \( \varepsilon \) is the engineering strain.

Viscoelastic creep is the elongation of the material in a time-dependent manner and this occurs when a continuous stress (e.g. IOP) is applied. Creep may be an important factor in the mechanical properties that arise in ectasia or conditions where corneal thinning occurs (Dupps and Wilson, 2006; Elsheikh, Alhasso and Rama, 2008b). A high modulus indicates a stiffer material or a lower compliance. Elastic materials that behave linearly have a constant elastic modulus whereas in non-linear materials such as the cornea and other biological tissues, the Young’s modulus is dependent on strain. Although for small strain levels, non-linear elastic materials can also deform linearly. The reported corneal Young’s modulus in the literature ranges from 0.1 to 57 MPa and it is thought that such a vast range of values is due to the variation in testing methods currently used (Garcia-Porta et al., 2014). The shear strength of the cornea is associated with the resistance of the stroma to sublayer bending and sliding. The interweaving collagen and other matrix forces provide the stromal resistance. Compared to the tensile strength, the shear strength of the cornea is found to be lower but plays a role in the transfer of tensile load between lamellae and this may have an effect on the shape of the cornea after photoablation treatments such as LASIK (Dupps and Wilson, 2006; Hatami-Marbini, 2014). Although it has several limitations, tensile testing is a standard method used for ex vivo elastic modulus determination of corneal biomechanical properties and other soft biological tissues. Other techniques include atomic force microscopy (AFM) or indentation, tonometry or inflation testing (Dupps and Wilson, 2006; McKee et al., 2011).

Several processes are involved in wound healing which include cell migration, cell proliferation, matrix deposition and tissue remodelling. The more important processes are cell proliferation and migration and they are driven and regulated by the release of certain growth factors (Yu et al., 2010). Contact inhibition, chemotaxis (movement of cells towards or against a concentration gradient), haptotaxis (directional motility or outgrowth of cells) and contact guidance have been shown to underlie directed cell migration. High levels of cell attachment and cytoskeletal contractility are associated with the migratory phenotype used by fibroblastic cells. Corneal fibroblast cells or keratocytes respond to changes in extracellular matrix (ECM) composition, stress and stiffness and these responses have an important part in how the cells react and respond to injury or surgery (Kim et al. 2012).
1.3. Cell migration and the mechanisms involved in cell motility

Cell migration is an important process involved in several physiological and developmental processes, including wound healing (Doyle et al., 2013). In addition to this, cell migration can also occur abnormally in adult life and this type of cell behaviour is related to pathologies such as invasion and metastasis of cancer. Cells can migrate in different ways and some cell types migrate individually whilst other cell types migrate as a collective. Collectively the cells can migrate as clusters, sheets or chains of cells (Aman and Piotrowski, 2010).

Cell guidance mechanisms can coordinate cell migration in processes such as wound healing, development, organ formation, and immune response. Directional cues such as soluble chemical gradients can be followed by cells, which is a process called chemotaxis. Durotaxis, is the ability of cells to follow gradients in stiffness of their ECM but is not as well studied as chemotaxis. This could be due to other factors such as pore size, coating density, surface topography and osmotic swelling of the substrate, that may affect experimentation of durotaxis (Roca-Cusachs, Sunyer and Trepat, 2013).

Cell migration involves two distinct types of forces that must be generated for cell movement. The protrusive force is required at the leading edge to extend lamellipodia or filopodia and the tractional force, which is needed to overcome the interactions that occur between the cells, the matrix and the adhesion between them (A. Kim et al., 2012). Physical forces at the cell–cell and cell–matrix interface can also guide cell migration but unlike chemical or stiffness gradients, physical forces can transfer directional information without needing a gradient. The influence of physical forces other than stiffness gradients on the mechanical guidance of cell migration enables the cells to coordinate their collective migration (Roca-Cusachs, Sunyer and Trepat, 2013). Tambe et al reported that by mapping the stress within a monolayer sheet, local cellular migrations were found to follow the local stress fields of maximal principal stress, within this stress landscape. However collective migration was found to follow a key principle that neighbouring cells in a monolayer transmit normal stress through the cell–cell junction but migration occurs along configurations of minimal intercellular shear stress (Tambe et al., 2011).

1.3.1. The cytoskeleton and cell dynamics

1.3.1.1. The components of the cytoskeleton and their role in cell motility

The overarching role of the cytoskeleton can be explained by three broad functions. The cell contents are organised spatially by the cytoskeleton, the cell is connected to the external environment both physically and biochemically through the cytoskeleton and it can move the cell or
change its shape by generating forces. The cytoskeleton consists of three main components; actin filaments, microtubules and intermediate filaments. These cytoskeletal polymers control the shape and mechanics of eukaryotic cells in a dynamic structure and are organised into networks within the cell. Actin filaments and microtubules can polymerise and de-polymerise, which creates forces that can drive changes in cell shape. All three components of the cytoskeleton can respond to external or internal mechanical forces and this can affect the organisation of the filaments within the networks. The key differences between the three cytoskeletal polymers in terms of network structure are stiffness, assembly dynamics, motor protein they interact with and polarity (Fletcher and Mullins, 2010).

The stiffest of the three cytoskeletal polymers are the microtubules. They can reorganise quickly, due to their complex dynamic state. During mitosis of a cell, the microtubule cytoskeleton rearranges to form the mitotic spindle, in preparation for cell division. Actin filaments are less stiff when compared to microtubules, but the actin filaments can be cross-linked into highly organised, more rigid structures such as bundled networks, branched networks and isotropic networks. Aligned actin filament bundles support filopodial protrusions, which are involved in cell to cell communication and chemotaxis. The polymerisation of actin filaments is necessary for advancing the leading edge of a migrating cell (Figure 5). Contractile actin filament bundles are known as stress fibres and the assembly of these structures occurs when cell surface adhesion molecules called integrins are stimulated (Pollard and Borisy, 2003; Naumanen, Lappalainen and Hotulainen, 2008). Intermediate filaments have the lowest stiffness compared to microtubules and actin filaments. They can be cross-linked to other intermediate filaments and to microtubules and actin filaments. They are assembled in response to mechanical stresses by many cell types but they are non-polarised (Fletcher and Mullins, 2010).

The cytoskeleton is an important structure and gives the cell rigidity. As the cell moves it will change its internal organisation because the cytoskeleton exhibits pre-stresses. The dynamics of a motile cell are controlled by mechanical structures such as the actin cytoskeleton, the cytosol and the plasma membrane. The actin cytoskeleton is a network of filaments that are viscoelastic and generate forces by polymerisation or through myosin motor activity (Abu Shah and Keren, 2013). Actin filaments provide internal mechanical support along with microtubules and intermediate filaments, which are other cytoskeletal polymers. Stable actin filaments are formed from the polymerisation of actin monomers into a helical arrangement of subunits. This polymerisation of actin filaments also drives the movement of cells in a crawling motion (Pollard and Cooper, 2009). Actin filaments are essential for cell locomotion and the formation of actin filaments from their monomeric subunits is carried
out by Arp 2/3 – mediated cross linking and branching of the lamellipodial actin network. This change in actin filaments at the leading edge of a migrating cell allows lamellipodia formation and directional movement, as shown in Figure 5 (Pollard and Cooper, 2009; Stricker, Falzone and Gardel, 2010; Akhshi, Wernike and Piekny, 2014).

**Figure 5** – A schematic diagram showing the mechanisms and forces involved in cell migration.

The interactions of actin with myosin motor proteins produce different types of movement. Firstly, myosin creates force between the actin filaments and this produces contractions that pull up the rear of the moving cell. It also produces the force to separate dividing cells into two and cells use a contractile ring consisting of actin filaments and myosin –II. Myosin can alter the shape of cells to form tissues. Secondly, cargo can be moved along actin filaments over short distances by myosin that is associated with subcellular organisms and macromolecular protein complexes (Pollard and Cooper, 2009).

The other components of the cytoskeleton, the microtubules and intermediate filaments, have different roles compared to the actin filaments but cell shape and mechanics are determined by the crosstalk or interplay of the three cytoskeletal networks (Huber et al., 2015). Microtubules interact with the actin – myosin cytoskeleton to polarise cells and to communicate cell shape changes and cell migration. Microtubules provide structural support to cells and cortical F-actin can be regulated through changes in microtubule stability by activating or inhibiting different Rho GTPases. At the leading edge of a motile cell, stable microtubules help to activate Rac to form lamellipodia and depolymerising microtubules help to activate RhoA to form stress fibres at the rear of the cell. The
feedback between the actin - myosin cortex and microtubules allows polarisation of cells for particular processes such as cell division, cell fate determination and cell migration (Akhshi, Wernike and Piekny, 2014).

Intermediate filaments are important for maintaining cell and tissue integrity and in the response of cells to different stresses. They resist mechanical forces by serving as intracellular ligaments and tendons and are abundant fibrous elements within a cell, capable of impacting cell migration from a mechanical and regulatory perspective (Pollard and Cooper, 2009; Chung, Rotty and Coulombe, 2013). The intermediate filaments can have a direct and indirect role in cytoskeletal rearrangements, cell mechanical properties, intracellular signalling and cell adhesion (Leduc and Etienne-Manneville, 2015). Certain intermediate filament proteins such as vimentin, promote cell migration and invasive cell behaviours. Other intermediate filaments such as keratins exert a more varied impact on processes such as cell migration (Chung, Rotty and Coulombe, 2013; Leduc and Etienne-Manneville, 2015). Intermediate filaments also depend on other partner proteins to enable their assembly, organisation, function and regulation. The Rho family of small G proteins are key regulators of the reorganisation of actin and microtubules and can also dramatically influence intermediate filament organisation. Adhesive structures of the cell such as desmosomes interact with intermediate filaments like vimentin and this suggests that intermediate filaments have direct control over focal adhesion dynamics (Leduc and Etienne-Manneville, 2015). The intermediate filament network tends to be reorganised around the nucleus or at the trailing end of an actively migrating, polarised cell. From a cellular mechanics viewpoint, intermediate filaments do impact cell migration and it is the mechanical signals that produce polarised cell protrusions and directed cell migration. The attachment of epithelial cells to the ECM is also associated with mechanosensing and mechanotransduction, involving the intermediate filaments that act as stretch detectors (Chung, Rotty and Coulombe, 2013; Leduc and Etienne-Manneville, 2015). Intermediate filament network integrity is critical for cell migration and invasion and there is growing evidence to suggesting a robust link between all three cytoskeletal sub-systems during different cellular processes that are important in cell function like cell motility and mechanosensing (Chung, Rotty and Coulombe, 2013; Huber et al., 2015).

1.3.1.2. Cell adhesion, the effects of mechanical properties of the cell and mechanotransduction

The mechanical properties of the cellular micro-environment can direct several important cellular processes such as cell spreading, migration and differentiation. To understand how cells integrate environmental cues to modulate their behaviour, mechanical properties as well as chemical signals must both be considered (Maskarinec et al., 2009). Adherent cells can attach to a substrate that can
vary in stiffness from soft to rigid material and can also vary in thickness and topography (Discher, Janmey and Wang, 2005). In order to achieve the correct organisation of cells and production of extracellular matrix (ECM), cell movement is guided by signals from the surrounding environment. Migration in response to chemical gradients and more recently in response to the influence of substrate or ECM stiffness has been investigated. It has been shown that cell dynamics and adhesion structures are affected by the stiffness of a substrate and cells have enhanced movement on stiffer and more strained substrates. This also has an effect on how cells proliferate and differentiate as well as cell movement (Moreo, Garcia-Aznar and Doblaré, 2008).

There are several proteins in the cell that have mechanosensitive functions and these proteins have been shown to be dependent on force in order to integrate into mature cell – matrix adhesions such as focal adhesions. Examples of these proteins are zyxin, vinculin and talin and transmission of force is just as important as these mechanosensitive proteins in order to further understand how force is transduced into a biochemical response (Schwarz and Gardel, 2012). Regarding cellular force generation during cell adhesion to the matrix, the actin cytoskeleton is the main component of the cytoskeletal network involved (Schwarz and Gardel, 2012). During cell migration, the actomyosin complex reorganises to form more closely aligned actin filaments to the front of the cell. The cell can pull on adhesion molecules (integrins), which are attached to the cytoskeleton and ECM, allowing cell movement to progress (Verdier, 2003).

The process by which a cell converts a mechanical stimulus into an electrical or biochemical signal is called mechanotransduction. There are two key processes involved in mechanotransduction; passive sensing and active sensing of mechanical stimuli. Passive sensing is when a cell reacts to an external force such as shear stress, compression, and extension. Active sensing occurs when an internal force is generated to measure the extracellular environment, which enables the cell to respond to changes in surface topography, stiffness and ligand density. These changes are sensed by cell traction forces, that stimulate an active response within the cell. Mechanical stimulation conversion into biochemical signals requires focal adhesions, which bind the ECM to the cytoskeleton and through this certain molecular signalling pathways are stimulated (Holle and Engler, 2011). The Rho/Rho-associated protein kinase (ROCK) signalling pathway is involved in the cytoskeleton and the contractility of cells. ROCK is an important regulator of actin organisation and therefore has an important part in cell migration. Following the binding of integrins to the ECM, guanidine exchange factors (GEFs) found near focal adhesions catalyse Rho GTPases including RhoA and Rac. The contractile force that the cell exerts is interpreted into ROCK phosphorylation through RhoA (Chen, Tan and Tien, 2004). ROCK affects several cellular processes once activated, including actin
organisation which occurs through LIM kinase (LIMK) and cofilin activation. The phosphorylation of myosin light chain (MLC) and the inactivation of MLC phosphatase stimulate cell contraction. A key role of this pathway is the upregulation and stabilisation of stress fibres in response to the generation of internal cellular forces and the interaction of the cell with the extracellular environment (Amano et al., 1996; Holle and Engler, 2011).

Another cellular mechanism for responding to mechanical forces that the focal adhesions are exposed to is through mechanosensitive channels that are hypothesised to function through a protein ‘gate’. These channels increase in permeability to soluble ions in response to contractile forces and are known as stretch activated channels. These mechanically activated ion channels are suggested to be the sensors of physical force. The transient receptor potential potential (TRP) family of proteins consists of six subfamilies of cation-selective channels and these channels are expressed in different tissues to mediate responses to physical and chemical stimuli. The TRP channels are permeable to calcium ions and it has been shown that mechanical stretching of cells causes transient calcium ion influx, which suggests they are involved in mechanosensing as stretch-activated channels. Changes in intracellular calcium ion concentration can affect several pathways and in particular, it increases contractility of stress fibres through MLC phosphorylation (Kobayashi and Sokabe, 2010; Holle and Engler, 2011). Another mechanosensitive channel known as the TREK-1 potassium channel has been investigated to further understand the mechanogating mechanisms of the so-called protein ‘gate’. TREK channels are polymodal potassium channels that are opened by chemical and physical stimuli such as stretching, cell swelling, voltage and heat (Martinac, 2004; Holle and Engler, 2011). TREK-1 expression has been observed in the central nervous system and in dorsal root ganglia neurons. Potassium currents were recorded in response to mechanical activation of dorsal root ganglia neurons in culture and it was found that this activity was lost in TREK-1−/− animals. This showed that TREK-1 expression is present in some mechanosensitive cells and is important for the detection of mechanical forces exerted onto the cell (Ranade, Syeda and Patapoutian, 2015).

1.3.1.3. Physical, mechanical and biochemical cues that can influence cell migration
Dynamic cell processes such as cell migration are accomplished through the integration of internal cues within the cell and external cues from the surrounding environment (Kurniawan, Chaudhuri and Lim, 2016). The ECM architecture is highly complex and varied and the physiological ECM is a key
regulator of several different cell functions (D. H. Kim et al., 2012). Substrate topography can influence the direction and speed of cell migration (Jeon et al., 2010). Changes in the surface topography, for example in the density of nano-patterned ridges, can modulate the speed and direction of cell migration in both individual and collective migration (Kurniawan, Chaudhuri and Lim, 2016). Another important factor affecting cell dynamics is matrix stiffness and the stiffness of tissues in vivo can range from Pa to MPa or GPa orders of magnitude (Discher, Janmey and Wang, 2005). It is also known that cells will migrate along a gradient of increasing stiffness, known as durotaxis. This is due to the ability of the cell to sense substrate stiffness though the cytoskeletal structures and associated proteins and adhesion molecules described previously (Moreo, García-Aznar and Doblaré, 2008; Roca-Cusachs, Sunyer and Trepat, 2013).

Along with physical and mechanical signals, the cells also secrete and respond to biochemical cues in the form of soluble factors such as matrix metalloproteinases (MMPs), chemokines and growth factors. These factors can all induce changes in the migratory behaviours of cells (Kurniawan, Chaudhuri and Lim, 2016). Chemokines are specialised signalling proteins or cytokines that are secreted from the cell and promote cell motility along a chemical concentration gradient and this process is called chemotaxis (Friedl and Wolf, 2010). MMPs are a key part of the mechanisms involved in cell migration through three-dimensional (3D) matrices surrounding the cells. For example, during wound healing, MMP-1 aids the motility of keratinocytes through the collagen matrix through the α2β1 integrin. It was found that the keratinocytes were unable to migrate through a mutant collagen gel that could not be cleaved by MMP-1 therefore highlighting the importance of collagen matrix degradation by MMP-1 to allow cell motility (Pilcher et al., 1997). Growth factors control the communication between the cell and the micro-environment of the cell and are soluble molecules released by the cell into its surroundings. There are several growth factors that are involved in the regulation of cell migration and some of the main factors include EGF, fibroblast growth factor (FGF) (Heinzle et al., 2012), PDGF (Shih and Holland, 2006) and transforming growth factor (TGF) – β (Kurniawan, Chaudhuri and Lim, 2016).

1.3.1.4. The ability of cells to sense mechanical change in their environment

Cells are able to sense mechanical changes in their environment and the means by which cells detect and react to these mechanical changes is known as mechanosensing (Moreo, García-Aznar and Doblaré, 2008). The connection between the surrounding substrate or ECM ligands outside the cell and the cytoskeleton within the cell is regulated by focal adhesions. These macromolecular structures physically form links between the cell and the ECM and are a critical part of the mechanosensing machinery (Grashoff et al., 2010). Integrins, a group of transmembrane receptors, are particularly important in mediating focal adhesion dynamics. They enable the cell to sense
adhesion through a mechanical interaction but also act as a signalling centre for several different regulatory proteins involved in a variety of cell processes (Ross et al., 2013). External forces acting on cells are transmitted between the ECM and the actin cytoskeleton through integrin – mediated adhesions. This mechanism is critical in transducing the effects of force to regulate cell function (Ross et al., 2013; Kurniawan, Chaudhuri and Lim, 2016).

The actin cytoskeleton is a highly dynamic network which can also mediate mechanosensing in cells (Blanchoin et al., 2014). Adherent cells are able to anchor to a substrate and can then exert contractile forces in order to explore their environment and move or respond to mechanical or chemical stimuli (Angelini et al., 2010). The interaction of actin in antiparallel orientation with the motor protein myosin causes cell traction forces (CTFs) also called actomyosin contractility (Ciobanasu, Faivre and Le Clainche, 2014). The contractile forces exerted by cells are generated through actomyosin interactions, which are part of the structure of filaments that form the cytoskeleton. The actomyosin contractile machinery transmits the forces generated and the cells exert traction forces to the underlying substrate or ECM through transmembrane proteins of the integrin family and focal adhesions (Moreo, García-Aznar and Doblaré, 2008). Actomyosin contractility aids in the maturation of focal adhesions and reinforces the anchoring of actin. It is also involved in the unbundling and depolymerisation of actin into individual filaments, which are important in actin re-structuring across the cell during cell movement (Wilson et al., 2010). Activation of different types of integrin can significantly change the traction forces generated by cells on 2D surfaces and can also affect the formation of ECM in 3D environments (Lin et al., 2013; Kurniawan, Chaudhuri and Lim, 2016). On 2D surfaces, actomyosin contractility adjusts the dynamics of cell adhesion, lamellipodial protrusions and retraction on the leading edge of the cell. Together these factors enable mechanosensing of the cell to its environment (Giannone et al., 2007).

1.3.1.5. Modes of cell migration
There are two broad modes of cell migration; individual cell migration and collective cell migration. Mesenchymal and amoeboid types of migration are examples of individual cell migration whereas collective cell migration is where junctions between cells are maintained so cells move collectively in clusters or as a sheet. The typical features seen in mesenchymal migration is an elongated cell shape, increased traction forces and cytoskeletal contraction that is highly polarised (Friedl and Wolf, 2010). In amoeboid migration cells have a more rounded appearance and this mode is used by highly motile cells. Amoeboid migration is connected to a reduced inherent polarity and focal adhesions in the cell, therefore allowing easier movement of cells despite differences in their surrounding matrices (Friedl, Borgmann and Bröcker, 2001).
Collective migration involves the movement of a group of cells and a key feature of this mode of migration is a strong polarity within the cells leading collective migration, which direct the movement of the rest of the cells through the ECM (Grada et al., 2017). On 2D surfaces, sheet migration occurs by the leader cells using lamellipodia at the leading edge and exerting traction forces through actomyosin contraction. An example of collective migration is the migration of epithelial sheets during wound healing in which cells move collectively along a basal surface (Kramer et al., 2013). In a 3D environment, during collective migration the leader cells extend structures that are similar to filopodia and pseudopodia to direct the movement of groups or clusters of cells that still maintain connections between cells in a more complex environment (Kramer et al., 2013; Miron-Mendoza et al., 2013). This mode of cell migration is regulated by several different cues due to the cell–cell interactions and communication that occurs during collective migration. Recent studies have found that factors such as the strength of adhesion between cells (Kabla, 2012), cell density (Duclos et al., 2014), contact inhibition of cellular movement (Desai et al., 2013), the traction forces exerted by the cell onto the substrate (Style et al., 2014) and the geometrical constraints from the tissue and the substrate that can regulate the formation of patterns in collective cell migration (Vedula et al., 2012; Doxzen et al., 2013).

Cells that are migrating can adapt and respond to the dynamic mechanical and biochemical changes that occur in their surrounding environment and can therefore change between the different modes of migration. Rho and Rac signalling and the balance between these two pathways mainly control the changes between mesenchymal and amoeboid migration (Bergert et al., 2012). Also mechanical properties of the microenvironment surrounding the cell such as matrix porosity, stiffness, confinement and density can play a key part in influencing the change between different migratory modes (Kurniawan, Chaudhuri and Lim, 2016).

1.3.1.6. Cell migration and interactions with the ECM during this process
Traction forces exerted by cells onto the ECM can alter the density, stiffness and architecture of the ECM surrounding the cell. The remodelling of the ECM matrix fibres surrounding the cell are done in an unequal, myosin–dependent way and this enables cell migration, correlating to the traction forces exerted by the cell (Bloom et al., 2008). Matrix degradation, cross–linking and deposition of new matrix are ways in which chemical remodelling occurs in the ECM. One of the key enzymes involved in the process of matrix degradation are the MMPs and the activity increases during tissue repair or cell invasion in cancer phenotypes (Kurniawan, Chaudhuri and Lim, 2016). Cell migration can be regulated by MMP activity, allowing cells to migrate along tracks created in the matrix (Sears and Kaunas, 2016).
As well as proteolysis of the matrix proteins, cross-linking within the ECM can promote cell migration by chemically remodelling the ECM. In tumour formation, the characteristic properties are ECM remodelling and stiffening. It is known that the stiffening of the ECM can improve cell growth and survival, promoting cell migration (Lo et al., 2000). Levental et al found that tumour formation was complemented by ECM stiffening, increased focal adhesions and heightened PI3K activity stimulated by integrins. This response was induced by collagen cross-linking and these factors promoted cell migration and breast tumour invasion (Levental et al., 2009). Cells are also able to secrete and deposit their own matrix and remodel the ECM through this mechanism. Hyaluronan, a glycosaminoglycan, is secreted into the extracellular space and is synthesised at the plasma membrane. In pancreatic cancer, hyaluronan production increases and it can be incorporated into the ECM. This was found to assist metastatic growth and cancer cell invasion or migration, enabling the increased hyaluronan production to be used as a marker in pancreatic cancer (Kultti et al., 2014).

Due to the continual remodelling of the ECM and matrix deposition, the stiffness of the ECM exerted initially on the cell will only have a temporary effect on cell behaviour due to the cell being able to remodel and change its microenvironment (Petersen et al., 2012). The dynamic interactions between the cell and the matrix regulate the tensional homeostasis between cells and their surroundings. The interactions between cells can affect cell migration along a stiffness gradient (durotaxis). If cell density increases to a level whereby neighbouring cells can transmit forces between them through cell–cell contact or via the elastic substrate, the effect of durotaxis is retracted and cell migration can occur freely across the stiffness gradient (Lo et al., 2000). The mechanisms within the cell that respond to changes in matrix mechanics change in order to remodel the ECM. The effects of mechanical loading on the synthesis of new matrix has been shown to impact at the gene and protein expression level within cells (Gupta and Grande-Allen, 2006; Popov et al., 2015).

Biological material such as the ECM tends to have unique mechanical properties when compared to materials that are often used as substrates for cell culture. Materials such as glass, plastic dishes and some gels such as polyacrylamide are deemed as simple materials because they have constant linear mechanical properties. Biological matrices that are fibrous networks often have non-linear mechanical properties, which mean that depending on the extent of deformation of the biological material, the stiffness measurements will change (Wen and Janmey, 2013). During cell migration, the anchorage of cells to the ECM is carried out by focal adhesions and organised by contractile stress fibres that are part of the actomyosin cytoskeleton and these structures are key in the dynamic
interactions between the ECM and the cell (Ciobanasu, Faivre and Le Clainche, 2014). In 3D micro-environments, it has been found that the anchored cells show major differences in their adhesion to cell differentiation and migration. This showed that the mechanical properties or stiffness of a substrate and the topographical information such as the dimension and shape are both equally important regulators of cell adhesion and the cytoskeletal structures involved and there is a need to further investigate these effects combined (Ochsner et al., 2010).

1.3.1.7. Different methods used to investigate cell migration in vitro

In the literature there are several commonly used methods for studying the cell migration and or invasive properties of cells. In vitro cell migration assays have several advantages over in vivo assays in that they are usually cost – effective, are relatively easy to conduct and can also be used for high-throughput testing (Kramer et al., 2013; Grada et al., 2017). Transwell migration assays also known as the Boyden chamber assay allows cell migration in response to chemical gradients to be investigated. The principle is based on two chambers containing medium that a separated by a porous membrane and the cells can migrate through the membrane, containing the correct pore sizes for the cells being investigated. Cell size can also be assessed due to the porous membrane and chemotaxis of cells can be measured using cell counts of cells in each chamber (Liang, Park and Guan, 2007; Kramer et al., 2013).

The wound healing assay or scratch wound is another in vitro assay used to investigate collective cell migration, which is a process that is critical in wound repair, immune responses, angiogenesis and cancer invasion and metastasis (Grada et al., 2017). This assay is a cheap and relatively straightforward method to investigate cell migration on 2D substrates and involves physically creating a “scratch” through a confluent monolayer of cells e.g. with a plastic pipette tip and imaging the wound closure over time. However, this method can be difficult to keep consistent and often the thickness of the scratch is uneven (Liang, Park and Guan, 2007; Kramer et al., 2013).

In order to gain more control over the gap created in a confluent cell layer, exclusion zone assays could also be carried out using silicone barriers. Cells wound be seeded with these in the well and in order to assess cell migration into the gap or exclusion zone, the barrier or stopper would then be removed and a clear area with cells seeded around it would be produced (Poujade et al., 2007). Other assays that are used include microcarrier bead assays and also the use of microfluidic chamber assays (Kramer et al., 2013; Carpi and Piel, 2014).
1.4. Use of different substrates for cell culture and how material properties affect cell behaviour

1.4.1. Substrates for biological applications

There are several different materials that are used for tissue culture and tissue engineering and as substrates for cell culture. Hydrogels are commonly used polymers that are able to adsorb large amounts of water without dissolving (Passos et al., 2016). They are formed from chemically or physically cross-linked polymer networks and were introduced as soft contact lens material using cross-linked poly (2-hydroxyethyl methacrylate (pHEMA) (Schacht, Van Vlierberghe and Dubrue1, 2011; Farandos et al., 2015). Natural polymers such as collagen, fibronectin or gelatin are known to have a tendency to adsorb proteins present in serum which could be a major problem for tissue engineered constructs (Schacht, Van Vlierberghe and Dubrue1, 2011). Biopolymers or biodegradable synthetic polymers have been used to produce tissue engineered vascular grafts (TEVGs) (Hasan et al., 2014). This was done by electrospinning a range of different polymers and this technique is commonly used to produce scaffolds. Electrospinning involves the stretching of a viscoelastic solution into nanofibers or microfibers using high electrostatic force (Hasan et al., 2014). The technique can also allow the fine-tuning of the mechanical properties and other characteristics such as the size, porosity and organisation of the material (Murphy, McDevitt and Engler, 2014).

Poly(vinyl alcohol) (PVA) is another polymer that is widely used as a biomaterial and due to many advantageous properties such as being biocompatible, hydrophobic, relatively cheap and non-toxic including thermal stability and good mechanical strength and flexibility (Karimi and Navidbakhsh, 2014). Due to these properties it has been tested as a potential biomaterial for biomedical applications including for contact lens material (Paradossi et al., 2003; Bhamra and Tighe, 2017). Poly(ethylene terephthalate) (PET) was also used in electrospinning applications to investigate its potential as a graft substitute for small diameter blood vessels as polytetrafluoroethylene (PTFE) and PET have been successful in treating large diameter arteries (Ma et al., 2005). PET has also been applied to soft contact lenses and was used in the investigation of an electrochemical sensor within the contact lens that could monitor the glucose concentration in artificial tear fluid. This could have the potential to continually monitor blood glucose levels and also could provide a way to monitor the IOP and detect early signs of glaucoma (Farandos et al., 2015). The different polymers discussed showed the flexibility and potential of polymers as biomaterials and further investigations into a silicone polymer which has also been applied in contact lens technologies previously called polydimethylsiloxane (PDMS) is discussed in Section 1.4.2.
1.4.2. Polydimethylsiloxane and its mechanical properties

1.4.2.1. Applications of polydimethylsiloxane

Polydimethylsiloxane (PDMS) is a silicone elastomer and its main application is the embedding or encapsulation and protection of electronic components as it has good dielectric properties and is a highly transparent, flexible elastomer that allows straightforward inspection of encapsulated components. PDMS successfully protects electrical/electronic components over a wide range of temperatures from -50 °C to 200 °C (Schneider et al., 2008; Dow Corning, 2014a).

There are several kits available commercially to prepare PDMS and a commonly used 2 part kit from Dow Corning is Sylgard® 184 Silicone Elastomer kit, consisting of a base elastomer and curing agent (Dow Corning, 2014a). This particular kit has been used in biological applications, especially investigations into cell mechanosensing and responses to changes in the mechanical properties of their surrounding environment or culture substrate (Palchesko et al., 2012; Xie et al., 2014; Fusco et al., 2015; Holle et al., 2017). PDMS has many desirable properties that make it a suitable substrate for biomedical applications. It is a biocompatible, thermally stable, durable and transparent polymer that is simple to handle and cost-effective (Mata, Fleischman and Roy, 2005; Sugiura et al., 2008). Biocompatibility is defined as the ability of a biomaterial such as a scaffold or matrix to act as a substrate that will support cellular function and facilitate molecular and mechanical signalling. Therefore the substrate used should not cause any adverse or undesirable effects on the cells or tissue (Kulkarni and Rao, 2013).

PDMS has been used for several decades as a biomaterial and for several biomedical devices such as contact lenses, catheters and prostheses (Quinn and Courtney, 1988; Abbasi, Mirzadeh and Katbab, 2001; Eleni et al., 2013). In the late 90’s, materials such as PDMS were used to produce contact lenses that had high oxygen permeability and were comfortable to wear by co-polymerising PDMS with other monomers. However the PDMS lenses were hydrophobic due to the presence of silicon and required treatment with wetting agents to make them more hydrophilic (Liu and Sheardown, 2005; Farandos et al., 2015). Rigid contact lenses were more durable and resistant to deposit build up than soft lenses but poly(methylmethacrylate) (PMMA) rigid lenses behaved as a glass at room temperature and therefore had negligible oxygen transfer. The main limitations of the rigid contact lens materials were that they had a restricted water content when compared to the human eye, impermeable to oxygen, which was essential for corneal epithelial cell survival and caused eye irritation. Since the introduction of soft contact lenses, they have been further developed to improve their oxygen permeability and wettability (Nicolson and Vogt, 2001; Farandos et al., 2015).
PDMS has also been used in other biomedical applications such as a coating for implants and the base material for micro-electrodes (Blau et al., 2011; Kim et al., 2011). Due to the ability to modify the mechanical properties of PDMS, it has also been used to simulate the properties of human tissue (Colombo et al., 2010; Payne et al., 2015). Other reported applications of PDMS are its use as a substrate or carrier for cells for example, as a scaffold in a bioartificial liver (Kataoka et al., 2005) and PDMS bioimplants for neuronal tissue reconstruction (Vaysse et al., 2015). Also, due to its ability to easily mold into sub-micrometre features, ease of bonding to itself and glass and high chemical resistivity, PDMS is most commonly used for the fabrication of microfluidic devices (Zhou et al., 2012) and MEMS (Schneider et al., 2008, 2009). PDMS has been increasingly used for soft lithography techniques such as micro-contact printing, replica molding and micro-molding in capillaries (Johnston et al., 2014; Placet and Delobelle, 2015). These techniques require the use of PDMS to create a mold that can incorporate microstructures for the transfer of patterns onto another substrate. The main advantages of PDMS for microfluidic systems over other substrate materials are that it is optically transparent down to 240 nm and it allows fast, simple fabrication. It also has low shrinkage rates and the high elasticity of PDMS can offer further advantages over traditional substrates materials that are rigid such as glass, silicon and harder polymers (Johnston et al., 2014).

1.4.2.2. Mechanical properties of polydimethylsiloxane

More recently, studies on the rheological and stress-strain properties of Sylgard® 184 made to the manufacturers’ guideline have shown that the material behaviour is elastic and it is assumed to be weakly compressible or incompressible following most analyses (Khanafer et al., 2009; Schneider et al., 2009; Johnston et al., 2014; Placet and Delobelle, 2015). The bulk mechanical properties of PDMS can vary depending on the temperature used for curing the polymer. Most applications of PDMS that have been reported do not have a standard temperature or duration for curing and therefore there is a lack of understanding of process-dependent variation in the mechanical properties of PDMS. Also curing temperatures above 200 °C have been stated as starting thermal decomposition of PDMS (Johnston et al., 2014). Testing of the bulk mechanical properties of PDMS has shown that a strain of up to 40 % produced a linear relationship between the Young’s modulus and working temperature. It also showed that applied strain had a minimal impact on the measured mechanical properties (Schneider et al., 2008).

Tensile testing is used to test the tensile strength of PDMS and the typical value of tensile strength reported in the manufacturers’ data sheet for Syglard® 184 is 6.7 MPa (Dow Corning, 2014a). However, there are no specific curing conditions and in the literature the tensile strength of PDMS ranges from 1-9 MPa (Schneider et al., 2008; Liu et al., 2009; Liu, Sun and Chen, 2009). Tensile testing can be carried out using the ASTM standard ASTM D412 test standard for vulcanised rubber.
and thermoplastic elastomers. The ASTM method is used to compare mechanical properties across different materials and is a precise enough test for the majority of materials (Liu et al., 2009). The mechanical properties of elastomers are different in that they can show elastic behaviour up to very high strain levels. At higher strain levels over 40%, the stress/strain curves obtained from the tensile stretch measurements will show a non-linear region before failure and ultimate tensile strength (UTS) of the material. Below 40% strain, a linear region on the stress/strain curve is observed and from this part of the curve the Young’s modulus can be calculated (Kim, Kim and Jeong, 2011). Metals which are solid state materials show reversible linear strain behaviour up to 1%. For materials that show a non-linear behaviour, the stress/strain dependence has to be corrected for by using higher order non-linear models such as the Mooney–Rivlin or Neo-Hooke model (Schneider et al., 2009).

Young’s modulus or elastic modulus $E$ is used to calculate the stiffness of a material. If a material is elastic it will return to its original shape when the applied stress is removed in a reversible manner. From the stress/strain curve obtained from mechanical tests, the slope of stress (force per unit area, Newton/m²) over strain, which is the elongation of a test specimen divided by the original length of the sample (dimensionless quantity), is calculated from a representative portion of the curve. Over a small range of stress, most soft biological tissues do show some linear elastic behaviour. However overall, their elastic behaviour is highly non-linear (Dupps and Wilson, 2006). Poisson’s ratio is a conversion factor that is used to relate transverse strain to longitudinal strain in the direction of elastic loading (Dupps and Wilson, 2006; Greaves et al., 2011). It has been observed that soft biological materials can exhibit a Poisson’s ratio between 0.3 and 0.5. PDMS is generally found to have a Poisson’s ratio of 0.5 as it is assumed to be an incompressible material (Lee et al., 2016).

In previous studies, the mechanical properties of PDMS have focussed on the different applications of PDMS such as thin membranes for sensors, biomedical, and the non-linear behaviour of PDMS in standard and modified preparations (Johnston et al., 2014). Liu et al showed that the Young’s modulus of PDMS membranes changes from bulk mechanical behaviour if the thickness is above 200 µm to being reliant on the dimension below 200 µm. The curing temperature used for PDMS was found to significantly affect thin PDMS films and their resulting mechanical properties (Liu, Sun and Chen, 2009). A commonly used PDMS kit is Sylgard® 184 from Dow Corning and is supplied in a two part kit with a base elastomer and curing agent, which is recommended to be used at a 10:1 (wt/wt) ratio of base elastomer to curing agent (Dow Corning, 2014a). Within the literature, differing ratios of base elastomer to curing agent have been investigated and this can cause changes in the mechanical properties of PDMS. By reducing the ratio of curing agent or cross-linker to the base elastomer, the resulting Young’s modulus of the PDMS decreases but this leaves free polymer which
can leach out. Therefore, the manufacturer recommends a particular ratio of the two components in order to maintain the stoichiometry of the process of cross-linking during gel formation (Palchesko et al., 2012).

1.4.2.3. The use of polydimethylsiloxane in the study of mechanobiology

PDMS has been used for important studies in mechanobiology and different cell behaviours in response to deformable substrates when attached to the surface, changes in mechanical stimulation of substrates and micro-structured substrates and how they affect key processes of attached cells (Kreutzer et al., 2014; Prauzner-Bechcicki et al., 2015; Bao et al., 2016). Palchesko et al prepared PDMS blends to be used as the basis for a tuneable system where the elastic modulus of the material could be adjusted to match a soft tissue type being investigated. By blending two different types of commercially available PDMS, Sylgard® 184 and Sylgard® 527, they could fabricate material with a range of different elastic moduli (Palchesko et al., 2012; Dow Corning, 2014a, 2014b). Biological validation of the different PDMS formulations was done by culturing a muscle cell line and a neuronal inducible – pheochromocytoma cell line with the different types of PDMS. It was found that the PDMS formulations supported cell attachment and growth and could probe the mechanosensitivity of the different cell types (Palchesko et al., 2012). The studies carried out in this thesis were based on the research carried out by Palchesko et al and the combination of the two types of Sylgard® were utilised throughout the work to produce 2D substrates with different mechanical properties.

Although it has many advantageous properties, PDMS requires further modifications to the surface for successful applications in biotechnology or biomedical applications. This is due to the hydrophobic, inert nature of the material and non-specific protein adsorption. Appropriate surface modifications for the PDMS are needed to increase surface wettability and produce a more hydrophilic surface, which will in turn decrease non-specific adsorption of proteins to the PDMS surface (Sugiura et al., 2008; Zhou et al., 2012). Various methods have been used to modify the surface of PDMS to make it more hydrophilic including oxygen plasma, UV, salinization, photo-induced graft-polymerisation and micro-patterning of the PDMS surface by chemically or topographically patterning the PDMS surface (Sugiura et al., 2008; Lee and Yang, 2012).

It was decided that PDMS would be used in the work discussed in the following chapters and it was found to be an appropriate culture substrate because it was an inert, biocompatible material lending itself to mechanobiology studies. The PDMS could be modified in terms of the bulk and surface mechanical properties and the effects of these material properties on cell interactions and behaviours would be investigated.
1.5. The effects of mechanical stimulation on cell behaviour: macroscale and microscale properties

Changes in cell behaviour have been observed in the literature in response to mechanical stimulation either from substrates of differing stiffness or by methods such as uniaxial stretching, compression, bending, substrate inflation tests and fluid shear stress (Brown, 2000). Three areas of mechanical tests can be identified to mimic the sort of physiological conditions cells may experience in vivo; hydrostatic pressure, fluid shear stress and substrate strain (Ursekar et al., 2014). For more physiologically relevant conditions, the strain applied should be biaxial as uniaxial strains may not reflect the physiological mechanical environment well enough. On the other hand cells are randomly orientated in normal cell culture conditions. Uniaxial forces have been shown to causes changes in the stress fibres and cell alignment but in vivo, there is more emphasis on cell to cell alignment and the generation of alignment – dependent effects (Ursekar et al., 2014).

Cell alignment plays an important role in several different cell behaviours such as cytoskeleton reorganisation and ECM remodelling. The micro-environment of a cell is surrounded by mechanical cues and these cues in vivo play an important role in tissue structure and function. The mechanical changes in the micro-environment can induce physiological changes in the ECM surrounding the cells (Li et al., 2014). One method to stimulate cells to align is through stretching a substrate or scaffold that the cells are cultured on to initiate these responses in the cells (Li et al., 2014). The loads applied can be at a physiological level for example, mechanical loading that mimics the physiological pressures or mechanical properties of arteries or veins or cardiac cells in vitro (Gupta and Grande-Allen, 2006; Colombo et al., 2010).

It has been shown that uniaxial cyclic stretching of cells, regardless of the cell type, causes the cells to align perpendicularly to the direction of stretch. This is also called the direction of minimal substrate deformation (Li et al., 2014; Kim et al., 2015). As discussed in Section 1.3.1.2, mechanotransduction of cells is strongly linked to the formation of focal adhesions and there are several mechanosensitive proteins or adhesion molecules that play a key role in the transduction of force (Schwarz and Gardel, 2012). The actin cytoskeleton is pivotal in this function of the cell and the forces felt by cells are critical for several processes such as cell differentiation and migration. It is also important to understand the cell mechanics on a nano or microscale to further understand the physical interactions and forces between the cell-matrix interactions (Sharfeddin et al., 2015).

The mechanical stimulation of mammalian cells has been shown to affect cell functions such as cell motility, proliferation and apoptosis. The cells response to mechanical stimulation also causes changes in protein and gene expression and this biochemical response to physical excitation of cells
is known as mechanotransduction (Steward *et al.*, 2010). Research carried out by Steward *et al.* (2010) used PDMS in a shear and stretching mechanical stimulation system. Morphological and structural changes induced by their device were investigated and quantified by examining cell shape and orientation in response to fluid flow or substrate stretching. They successfully developed a single device approach capable of stimulating cells through multiple modes of stimulation (Steward *et al.*, 2010).

AFM can be used to measure the mechanical properties of cells and their responses to changes in their microenvironments, by quantifying cellular forces. This method of mechanical force measurement is based on measuring the deformations induced by force from a sensor and converting this measurement to the true force values through its elastic properties. The force is assumed to be proportional to the deformation if the deformation is small and this relates to Hooke’s Law (Schoen *et al.*, 2010).

The nanotopography of a substrate can have a significant impact on cellular functions such as changes to the cell shape or cell differentiation. It was found that the nanotopography measured by AFM changed the expression of cytoskeletal and focal adhesion components as reflected by the mechanical properties obtained using AFM nanoindentation (Yim *et al.*, 2010). This showed that both the mechanical properties and the surface topography of a substrate have an influence on integrins bound to the surface and their expression, the assembly of focal adhesion molecules and F-actin organisation.
1.6. Conclusions

Following the broad literature review, the structure and function of the cornea and the effects on the biomechanics were discussed in terms of the corneal response to wound healing following injury or surgery and in some illnesses. Cell migration is a key part of corneal wound healing and is affected by the biomechanical changes in the cornea. Testing the mechanical properties of the corneal cells or the cornea tissue in vivo is a challenging task however there are non-destructive methods to characterise the corneal biomechanics. A key part of the wound healing process is cell migration and the factors involved in the regulation of cell migration related to the cytoskeleton, the interactions of the cell with a substrate or ECM and physical or chemical cues that can influence cell migration were discussed in detail. The major part of the cytoskeleton involved in mechanosensing is the actin cytoskeleton, in particular F-actin and the formation of contractile stress fibres. The forces acting on the cells are transmitted through the ECM by integrin – mediated adhesion molecules. The forces felt by individual cells or cells as a collective are important in further understanding the physical changes and responses of the cells to the differences in substrate stiffness and changes in surface topography as observed at the cellular level. The changes in surface topography and substrate stiffness can regulate cell behaviours such as cell alignment, cell differentiation and cell migration.

1.6.1. Research aims and objectives

The literature shows that the effects of mechanical changes to the cell and its surrounding environment are important in understanding changes in certain processes involved in cell functions such as cell adhesion and cell migration. Rabbit corneal epithelial (RCE) cells are used in this research to investigate the effects of mechanical stimulation on single cells. Substrates with differing mechanical and surface properties can be used to enable the further investigation of the RCE cell response to mechanical stimulation. Key cellular processes such as adhesion, proliferation and migration can be measured following cell culture on different substrates to compare the effects of substrate properties on the RCE cells. Also, the mechanical properties of single cells can be measured using atomic force microscopy to study cell responses to different substrate stiffnesses at the nanoscale level. Furthermore, the effects of an external mechanical stimulation to assess collective cell migration and single cell responses would allow the impact of mechanical changes in the extracellular environment to be measured in relation to changes in cell behaviour and morphology. More detailed research aims, objectives and hypotheses can be found in Chapters 3-6.
Chapter 2
Materials and Methods
2.1. Preparation of Polydimethylsiloxane

2.1.1. Preparation of Sylgard® 184

Polydimethylsiloxane (PDMS) was prepared using Sylgard® 184 (Dow Corning, Univar Speciality Consumables). The kit was supplied as two parts containing the elastomer base Silicone (Dimethylvinylated and Trimethylated Silica) and the elastomer curing agent (Silicone resin solution). The mixing ratio recommended by the manufacturer for the two components was 10 parts elastomer base to 1 part curing agent (wt/wt) (Dow Corning, 2014a). The amount of PDMS component required was calculated by weight in grams. This was weighed out by pouring the viscous elastomer base into a 90 mm plastic petri dish. To this weight (10 parts), one part of curing agent was calculated and added drop wise to the elastomer base to make up the total weight in grams, using a Pasteur pipette. It was found that 5 g of total weight of PDMS (both parts) produced PDMS of the correct thickness per petri dish (600-800 µm).

The two components were then mixed together vigorously in the petri dish using a clean spatula for at least 5 minutes of continuous mixing. The PDMS was transferred further into a clean plastic petri dish, covered with the lid and spread as evenly as possible across the base of the petri dish. The petri dish/dishes were then placed into a secondary container and kept at approximately 20 °C for 48 hours to cure. The PDMS membranes obtained after curing are transparent and have very few impurities throughout the thickness for imaging purposes.

2.1.2. Preparation of Sylgard® 527

Sylgard 184 and Sylgard 527 are primarily made from the same material and consist of dimethyvinyl-terminated dimethyl siloxane and dimethyl, methyl hydrogen siloxane therefore the polymer chemistry of the two types of Sylgard combined would not be altered significantly as they both consist of a basic siloxane chemistry (Palchesko et al. 2012). However, there are some differences in the compositions of the two different Sylgard kits. In addition to the two main siloxanes found in Sylgard 527, Sylgard 184 also consists of additional chemicals and silica nanoparticles. Sylgard 527 does not contain any fumed silica filler or other reinforcements and the presence of the fumed silica nanoparticles in Sylgard 184 significantly increase the stiffness of the polymer (Oláh et al. 2005; Dow Corning 2016a; Dow Corning 2016b; Dow Corning 2016c; Dow Corning 2016d). Therefore combining Sylgard 184 and Sylgard 527 could have an impact on the resulting mechanical properties of the PDMS blends, due to the composition, chemical reactions of each component, and the presence of the silica fillers and their distribution throughout the bulk of the material.
The preparation of PDMS membranes using Sylgard® 527 Silicone A&B dielectric gel (Dow Corning, Univar Speciality Consumables) was the same as Sylgard® 184 apart from the ratio of the two components, parts A and B as they were to be mixed in a 1:1 (wt/wt) ratio, as recommended by the manufacturer (Dow Corning, 2014b).

According to the manufacturer’s guidelines for Sylgard® 527, once the components were mixed the gel had a working life of 16 hours and a gel time of about 24 hours at room temperature (20 °C). However a curing time of 48 hours was decided as a standard time for room temperature curing of each type of PDMS to ensure the maximum curing time was reached for each PDMS. This was especially important for the different PDMS blends, as described in Section 2.1.3. Once cured, pure Sylgard® 527 was found to be a much softer polymer compared to blended PDMS samples described in Section 2.1.3. It was difficult to handle and to use in mechanical testing, as it would tear easily. The softer PDMS blends shown in Table 2, Section 2.1.3 (PDMS 1:5 and PDMS 1:10) were also challenging to handle and carry out further experiments with and therefore were not used in the majority of experiments.

2.1.3. Preparation of PDMS blends mixing Sylgard® 184 and Sylgard® 527

The two different types of Sylgard® described in Sections 2.1.1 and 2.1.2 of this Chapter were used to produce a range of different PDMS elastomers by blending both the pre-mixed individual kits of Sylgard® 184 and Sylgard 527® at varying ratios to each other before curing at room temperature (20 °C). This method produced a range of PDMS membranes with different bulk mechanical properties.

The PDMS blends that were prepared by combining Sylgard® 184 and Sylgard® 527 (once they were mixed individually as explained earlier) were made using weight in grams and using the following ratios explained in Table 2.
Table 2 - Table to show the different PDMS blends and the mixing ratios of Sylgard® 184 and Sylgard® 527 used.

<table>
<thead>
<tr>
<th>PDMS Blend Ratios (184:527)</th>
<th>10:1</th>
<th>5:1</th>
<th>1:1</th>
<th>1:5</th>
<th>1:10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sylgard® Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>184/g</td>
<td>4.5</td>
<td>4.5</td>
<td>2.25</td>
<td>0.9</td>
<td>0.45</td>
</tr>
<tr>
<td>527/g</td>
<td>0.45</td>
<td>0.9</td>
<td>2.25</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Final Combined Weight/g</td>
<td>4.95</td>
<td>5.4</td>
<td>4.5</td>
<td>5.4</td>
<td>4.95</td>
</tr>
</tbody>
</table>

The PDMS blends produced using these 2 particular types of Sylgard® were based on a paper by Palchesko et al (2012). It has been shown that by producing different PDMS formulations, the mechanosensitivity of different cell types can be studied using these substrates. The elastic moduli of the different PDMS blends could be fine-tuned to match that of soft tissue, allowing the mechanobiology of different cell types to be investigated further (Palchesko et al., 2012; Feinberg, Funderburgh and Simko, 2015). The PDMS blend 10:1 of Sylgard® 184: Sylgard® 527 was used in the research carried out in this thesis and was different to what Palchesko et al had used (PDMS blend samples were used for research purposes only). However other blend ratios were kept the same in order to maintain a good range of PDMS materials with differing stiffness.

The same methods of preparation for the individual Sylgard® kits as described in Sections 2.1.1 and 2.1.2 were used to prepare the PDMS blends shown in Table 2. Following the curing period, each different blend of PDMS was cut into 2 cm² sections using a sterile scalpel to be used for further analysis. Once cured the normal range of thickness obtained with the PDMS blends was between 600-800 µm and the cured samples were handled using tweezers. The PDMS blends were prepared as they were required and were only stored at room temperature once cured for no longer than one month. Cut PDMS sections were also stored in 90 mm plastic petri dishes until required.
2.2. Cold Atmospheric Pressure Plasma Treatment of the PDMS Surface

Plasma treatment was used on the surface of the cured PDMS blends to produce a more hydrophilic surface to encourage cell attachment. The plasma source used was a cold atmospheric pressure (CAP) dielectric barrier discharge plasma jet. The plasma was driven by an in-house built 25kVpp - 15 kHz power supply and the power delivered to the plasma was limited to <5W. Plasmas have been used for many years for sterilisation of medical equipment, packaging in the food industry and implants (Kong et al., 2009; Pankaj et al., 2014). CAP (less than 40 °C at the point of application) have been developed to potentially provide a plasma that can be used on living tissue (Kong et al., 2009) and atmospheric plasma is a low cost method that is easily implemented (Liu et al., 2010). The jet consisted of a quartz tube with an inner diameter of 1.5 mm and a metallic electrode wrapped tightly around the quartz tube. The ground electrode was situated underneath the PVC sample holder and the plasma was driven by an in-house built half-bridge resonant power supply.

The gas flow used through the jet was typically 3 standard litres per minute (SLM) of helium with a 0.5 % admixture of oxygen (15 standard cubic centimetres per minute (SCCM). The admixture of helium and oxygen combined the high thermal conductivity of helium and the oxidative properties of oxygen-derived species and could also be used at lower temperatures (Liu et al., 2010). The jet operated in open air and although some air may have diffused into the plasma, reactive oxygen species were expected to be the main reactive species in the system. The jet was scanned across the PDMS substrate and a programmable XY-translation stage was used to ensure even and repetitive treatment of PDMS samples. The jet described a grid pattern at 1 mm divisions at a speed of 40 mm/s. Two scans were performed per sample and the plasma treatment took around 40 seconds to be completed for a 2 cm² PDMS sample size. The plasma jet was in direct contact with the PDMS sample surface for the 40 second treatment.

Prepared PDMS samples with surface area of 2 cm² were treated on one side using the CAP plasma. The sample was placed flat in a clean petri dish that fit inside a petri dish holder on the moveable stage. The jet scanned across the surface of the PDMS substrate and a programmable XY-translation stage was used to ensure even and repetitive treatment of PDMS samples (Figure 6).
2.2.1 Plasma treated PDMS transportation and sterilisation

Immediately after CAP treatment of the PDMS substrate, the treated samples were placed into deionized (DI) water in a Duran bottle using tweezers. It has been suggested that by keeping the samples immersed in pure water immediately after plasma treatment, the plasma treatment is preserved in particular, oxygen plasma treatment (McDonald and Whitesides, 2002; Mata, Fleischman and Roy, 2005). It was also shown that the longer the plasma treated samples were exposed to air for, the greater they recovered the hydrophobicity of the PDMS surface (Lawton et al., 2005; Mata, Fleischman and Roy, 2005). The PDMS samples were then transported in the Duran bottles to be autoclaved on a liquid cycle at 121 °C for 1 hour. This was done to heat sterilise the PDMS in pure water whilst maintaining the plasma treatment effects on the surface of the PDMS. In the literature it has been found that very high curing temperatures can change the mechanical properties of the PDMS. Liu et al (2009) found that the temperature at which thermal decomposition of PDMS occurs is 310 °C, which was found in other reports too. They concluded that longer curing times at temperatures exceeding 200 °C the mechanical properties of PDMS will be reduced (Liu, Sun & Chen 2009). This showed that by heat sterilising the PDMS at 121 °C would not affect the mechanical properties of the PDMS further and the PDMS was already cured at room temperature at this point. After the sterilisation step, the PDMS samples (untreated and plasma treated) were handled within a biological safety cabinet (BSC) using sterile tweezers.
The samples were transferred out of the DI water using sterile tweezers and into sterile cell culture treated well plates, within the BSC. The plates were then left in the BSC on a UV cycle for one hour, which added an additional sterilisation step and also allowed some evaporation of pure water from the samples although the samples were kept covered. After this the sterilised, plasma treated and untreated PDMS was ready to be used for further experiments. The method of plasma treatment of the surface of PDMS produced a more hydrophilic surface that promoted cell attachment. The flow diagram in Figure 7 summarises the processes used to generate sterile PDMS samples for rabbit corneal epithelial (RCE) cell culture.

![Flow diagram showing the process used to produce sterilised PDMS for culture with RCE cells.](image)

### 2.2.2. Wettability

The wettability of PDMS surface following CAP plasma treatment was investigated by quantification of the water contact angle using the OCA20 goniometer (DataPhysics, Germany). Contact angle measures the wettability of a surface and was used to measure how hydrophobic or hydrophilic the surface of the PDMS was before and after plasma treatment. This measured the angle formed between the liquid/solid interface and the liquid/vapour interface of each droplet to give the water contact angle.

The contact angle was measured by adding a single 1 µl droplet of DI water to the surface of the PDMS and imaging the whole droplet by capturing the profile of a pure liquid on a solid surface. Once the image was taken, the water contact angle was measured by processing the images using the SCA20 software. If the surface was hydrophobic, the droplet would not spread over the surface.
and therefore the water contact angle would be high. If the surface was hydrophilic, the droplet would spread over the surface of the PDMS and the water contact angle would therefore be lower.

Surfaces that are hydrophilic are able to interact with water molecules. A hydroxylated surface (OH-terminated) is formed on highly reactive hydrophilic surfaces from the dissociation of water molecules, hence the ability of water molecules to spread across the hydrophilic surface. However a less reactive hydrophilic surface would interact with water molecules through hydrogen bonding, whereby the water molecules do not dissociate. Hydrophobic surfaces are less likely to bind to water molecules. Hydrated ions that subsequently bind to the surface form a water shell and this allows small proteins from fluid, serum or coating to adsorb to the hydrophobic surface. Protein adsorption on hydrophilic surfaces occurs between the hydrophilic amino acid side chains of the protein and the water layer on the surface, through polar and ionic interactions (Michaelis, Robelek and Wegener, 2012). Polar and hydrophilic surfaces have a higher affinity to protein binding and have an important role in cell adhesion and cell growth (Poncin-Epaillard et al., 2012).

For the PDMS samples, the water contact angle was tested for untreated PDMS, untreated PDMS after autoclaving, PDMS following plasma treatment, and PDMS after plasma treatment and autoclaving. These experiments were performed in an unsterile environment and therefore the samples were discarded after carrying out the contact angle measurements. The resulting contact angle measurements are an average of 3 samples per condition with 2 measurements per sample at each time point. For each measurement the mean of 2 angles was used per sample with an n number of 3.

2.2.3. Surface chemistry

Untreated and plasma treated PDMS samples were analysed using the K-Alpha™ X-Ray Photoelectron spectrometer (XPS) system (Thermo Scientific, UK). A survey scan was carried out on three areas on each PDMS sample to determine the elemental and chemical composition on the surface of untreated and plasma treated PDMS. High resolution scans on silicon, carbon and oxygen were also carried out as they were the known components of the PDMS and oxygen plasma treatment. The high resolution scans were also done on the three areas on each PDMS sample. The binding energies observed were cross referenced to binding energies of functional groups known to be present on the surface of PDMS using The Handbook of X-ray Photoelectron Spectroscopy (Moulder et al, 1992).

Area maps across a representative PDMS sample were carried out to show the distribution of the elements of interest over the whole surface before and after plasma treatment. These were
snapshots of a 5 mm x 5 mm area of the PDMS sample. PDMS samples were prepared as previously
described in Section 2.1.3 and after 48 hours curing, small sections of PDMS were cut using a clean
scalpel. Plasma treatment was carried out following the methods described in Section 2.2 of this
Chapter, but samples were tested in the XPS equipment as soon after the plasma treatment as
possible. It was ensured that the samples were kept clean and dry before the experiment and were
disposed of after the measurements were carried out therefore the steps detailing plasma treated
PDMS sterilisation were not necessary for these measurements. For each sample, three points were
measured and the results were analysed using the Thermo Scientific Avantage™ data system.

2.3. Mechanical Testing of PDMS

2.3.1. Tensile testing

Tensile testing of PDMS blends was carried out using the Instron 3366, a bench-top, computer
controlled dual column tensometer or universal testing machine (Instron, UK). The maximum load
capacity was 10 kN and the maximum test speed was 500 mm/min. The 6 different types of PDMS
used as described in Table 2 (Section 2.1.3) were tested using this instrument and 3 samples were
tested for each PDMS blend (n=3). The PDMS samples were cut into rectangles of 80 mm in length
and 25 mm in width with a gage length of 30 mm. The thickness of the PDMS blends had previously
been measured and they ranged between 600 – 800 µm. The test speed or extension rate was set to
254 mm/min. The samples were tested using ultimate tensile strength (UTS) until failure of the
PDMS samples or when they broke. The extension rate was chosen based on the crosshead velocity
used in the literature previously as part of several experiments to characterise the bulk mechanical
properties of PDMS (Johnston et al., 2014).

Tensile testing carried out by Johnston et al was performed to the American Society for Testing and
Materials (ASTM) International standards and also used a universal testing machine. The ASTM
method for tensile testing is the standard used to compare mechanical properties of different
materials and is suitable for most materials. However, some materials such as elastomers can exhibit
elastic behaviour even at high strain levels (Johnston et al., 2014). The same test speed was used in
tensile tests carried out on PDMS blends. The data obtained from the experiments included load,
extension, tensile strain and tensile stress. Figure 8 shows a typical load/extension curve obtained
from tensile testing of PDMS samples.
Figure 5 – Representative load extension curve for PDMS 184. Tensile testing was carried out on all samples to their ultimate tensile strength (UTS) or failure of the PDMS sample.

The UTS of the PDMS sample or the failure point was measured as well as the engineering stress and strain for these experiments. The Young’s modulus was calculated from the engineering stress strain curves obtained from the tensile testing data. The Instron 3366 software calculated the tensile strain (mm/mm) by dividing the extension (mm) by the gage length of the PDMS (30 mm). From the stress strain curve, Young’s modulus can be calculated from the linear portion of the curve (Liu et al., 2009). A representative engineering stress strain curve is shown in Figure 9. This showed the data obtained from tensile testing of PDMS 184 and the data range plotted on the graph was limited to 0.3 strain. By limiting the data to this strain level, a linear fit equation could be used (Excel) to obtain the stress and strain values and calculate the Young’s modulus, as in the literature it has been shown that at lower levels of strain below 40 %, the elastic linear region can be used for calculations of Young’s modulus (Kim, Kim and Jeong, 2011; Johnston et al., 2014; Lee et al., 2016). The area of the slope used for deriving the engineering stress and strain to be used in Young’s modulus calculations has been highlighted in Figure 9 on the representative curve.
The stress and strain values were used from the linear part of the graph as shown in Figure 9 in order to calculate the Young’s modulus using the following equation:

\[
E = \frac{\sigma}{\varepsilon} \quad \text{Equation 2 – Young’s modulus (Beer, 2012)}
\]

In the Young’s modulus equation, \( E \) is Young’s modulus, \( \sigma \) is the engineering stress and \( \varepsilon \) is the engineering strain. Equation 2 is derived from Hooke’s law which describes the linear relationship between stress and strain in relatively small material deformations (Beer, 2012).

![Figure 6 – An engineering stress strain curve for PDMS 184 showing a representative linear fit with data rate limited to 0.3 strain.](image-url)
2.3.2. Electronic speckle pattern interferometry (ESPI)

Another method of mechanical testing was carried out using a laser interferometry technique, using a rig and method designed by Abby Wilson as detailed in her PhD thesis titled “An Investigation into the use of Laser Speckle Interferometry for the Analysis of Corneal Biomechanics” written by Abby Wilson© (Wilson, 2017). ESPI is a technique that generates displacement in the sample and gives a value of maximum displacement. The experiments were also conducted by Abby Wilson and the raw data was processed by Abby Wilson to obtain the maximum deflection data. ESPI has been used for several years in the engineering industry to quantify the mechanical properties of materials, detect structural differences within the material and predict failure models (Zhang et al., 1998; Wilson, Marshall and Tyrer, 2016).

Laser interferometry is also a useful tool for quantifying the mechanical properties of soft biological tissue such as the cornea as it is a non-destructive real time method and can be adjusted to physiological pressures as it is highly sensitive (Wilson, Marshall and Tyrer, 2016). In addition to this, the mechanical response to loading in terms of the displacement can be measured across a whole surface enabling an improved understanding of the mechanical responses of certain biological tissues (Wilson, Marshall and Tyrer, 2016). Hence why ESPI was used on the PDMS blends and this allowed a much more sensitive and appropriate technique to be used that considered the material properties and elastic characteristics of the PDMS and also the thickness of the sample (Wilson, 2017).

For the work carried out in this thesis, the maximum displacement values were then used as shown in Equations 3 – 6 (Roark and Young, 1989);

Firstly hydrostatic pressure was used within the rig (rig design and methodology by Abby Wilson), where $\rho$ was the density of fluid in this case water, $g$ was gravity and $\Delta h$ was the height change from the baseline.

\[ P = \rho g \Delta h \quad \text{Equation 2 – Hydrostatic pressure.} \]
Assumptions were made about the PDMS sample and it was assumed to behave as a circular flat plate with uniform load and a fixed boundary. For this calculation, the maximum stress and deflection for a loaded flat plate were used as shown in Equations 4 and 5. Where \( \sigma_m \) is the maximum stress (N/m\(^2\)), \( p \) is the uniform surface pressure on the plate (N/m\(^2\)), \( r \) is the radius of the circular plate (m), \( Y_m \) is the maximum deflection, \( D \) is flexural rigidity (E.t\(^3\)/12(1-\(v\))), \( E \) is Young’s modulus of elasticity (N/m\(^2\)) and \( t \) is plate thickness (m).

\[
\sigma_m = \frac{3pr^2}{4t^2} \quad \text{Equation 4 - At edges of the plate}
\]

\[
Y_m = \frac{pr^4}{64D} = \frac{0.171pr^4}{E.t^3} \quad \text{Equation 5 - At the centre of the plate}
\]

Therefore to find \( E \) the equation became;

\[
E = \frac{0.171pr^4}{Y_m.t^3} \quad \text{Equation 6 - Young’s modulus using maximum deflection.}
\]

The pressure (\( p \)) used was 7.35 Pa and the thicknesses (m) of the PDMS samples used for ESPI were measured using the Talysurf CLI 2000 (Taylor Hobson\(^\text{®} \), UK). The average thicknesses used are detailed in Table 3 below. The radius (\( r \)) used was 6.5625 mm or \( 6.56 \times 10^{-3} \) m and the maximum deflection was obtained from experiments and data processing carried out by Abby Wilson, given in millimetres (Table 4, Chapter 3, Section 3.2.2.2). All units were converted to metres for calculations and the results are discussed in Chapter 3 Section 3.2.2.2.
Table 3 – Thickness measurements of PDMS blends used in ESPI Young’s modulus calculations converted to metres for use in calculations (average values, n=3, ±SD).

<table>
<thead>
<tr>
<th>PDMS</th>
<th>Average Thickness (µm)</th>
<th>Average Thickness (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>184</td>
<td>839.81±227</td>
<td>8.3981 x 10^-4</td>
</tr>
<tr>
<td>10:1</td>
<td>756.16±247</td>
<td>7.5616 x 10^-4</td>
</tr>
<tr>
<td>5:1</td>
<td>683.18±163</td>
<td>6.8318 x 10^-4</td>
</tr>
<tr>
<td>1:1</td>
<td>789.04±251</td>
<td>7.8904 x 10^-4</td>
</tr>
</tbody>
</table>

2.4. Atomic Force Microscopy

2.4.1. Preparation of Polydimethylsiloxane samples for AFM measurements

PDMS samples were used in experiments as described previously in Sections 2.1, 2.2 and 2.2.1 of this Chapter. The blank control PDMS samples were ready following sterilisation of the PDMS and were stored in phosphate buffered saline (PBS) (1X, without Ca and Mg, Lonza) at 4 °C. The cell seeding density used for each PDMS sample in the RCE cell–PDMS condition was 8.421x10^3/cm^2. RCE cell culture has been described in more detail in Section 2.5 of this Chapter and Section 2.6 describes RCE cell culture on PDMS blends. RCE cell seeded PDMS samples were cultured for 24 hours at 37 °C 5 % CO₂ in 2 ml RCE cell culture medium. The RCE cell–PDMS samples were then fixed using a 3.7 % formaldehyde solution following the method described, Section 2.6.1. Samples were stored at 4 °C in PBS until required. For the condition of RCE cells on tissue culture plastic, the same seeding density of RCE cells was used and cell suspension containing the correct concentration of cells was added to a sterile 40 mm tissue culture dish (TPP®, Switzerland) containing 2 ml RCE cell culture medium and cultured for 24 hours at 37 °C 5 % CO₂. After this period the RCE cells were fixed and stored in PBS at 4 °C until required.

Four different PDMS samples were prepared and measured, which were PDMS 184 (Sylgard 184 only), and PDMS blends PDMS 10:1, PDMS 5:1 and PDMS 1:1 (ratio of Sylgard 184: Sylgard 527) as described in Section 2.1. When the fixed RCE cell–PDMS samples were needed for AFM measurements, one sample was transferred into a 40 mm tissue culture dish that was the correct fit for the petri dish holder on the AFM stage. This was done using tweezers and 2 ml of fresh PBS was added to cover the sample. The dish was then ready to be placed on the AFM stage once the equipment had been assembled and the experimental parameters were set.
2.4.2. Atomic force microscopy using the JPK NanoWizard® 3 BioScience AFM

2.4.2.1. Experimental parameters

The JPK NanoWizard® 3 BioScience AFM (JPK Instruments, Germany) was used to perform mechanical measurements on RCE cells attached to tissue culture plastic and to different PDMS blends, and also surface mechanical properties of PDMS blends itself. These conditions were used to investigate the effects of PDMS with different mechanical properties on RCE cell mechanics and cell attachment. Training and support were provided by Dr Alex Winkel, senior applications scientist, JPK Instruments Ltd.

The JPK NanoWizard® 3 was mounted onto a Nikon Eclipse Ti inverted microscope before beginning the experiments. For all conditions the HQ: CSC37 non-coated silicon cantilevers were used (MikroMasch, Germany). Cantilever B was used in all experiments as the chips came with three different types of cantilever. This AFM cantilever had a resonance frequency ranging from 15-30 kHz and a force constant ranging from 0.1-0.6 N/m, according to the manufacturer data sheet and was used for both PDMS samples and RCE cell–PDMS samples throughout experiments. The length of the cantilever was 350 µm ± 5 µm with a conical shaped tip. This particular cantilever was used for the experiments because it is a general purpose cantilever with a relatively low force constant. Imaging and nanomechanical measurements were carried out on samples using QI™ mode over a 50 x 50 µm area at a resolution of 128 x 128 pixels. This size of area was chosen in order to image a single cell in one scan and to ensure that all parts of that cell were included in the measurement. The resolution was found to be suitable for producing clear images of an RCE cell within a reasonable time period.

The following parameters were set for QI™ mode measurements: z length = 3 µm, imaging speed=15 µm/s. After manually changing the z length, the other imaging parameters were automatically changed in the JPK SPM software (JPK Instruments, Germany) in relation to the z length. The z length of 3 µm was chosen to give enough clearance for the height of the expected sample feature and the additional retract from the tip. The optical image of the samples and AFM cantilever can be seen using the CCD camera and the JUnicam software, which are recognised automatically within the JPK SPM software once connected. All measurements were carried out at room temperature in liquid.

Due to the duration of each scan, fixed RCE cell-PDMS samples were used. At the resolution used, one scan in QI™ mode would take over 2 hours to complete. Three QI™ mode scans were planned...
per sample (contained in a petri dish) using the equipment, which meant that live cells could have been out of their controlled incubator environment for a minimum of 6 hours. This did not include equipment issues that may have caused further delay of the experimental plans. This would be detrimental to the live RCE cells and therefore could have affected the quality and reproducibility of the data obtained as only one scan could be performed at a time. Therefore using fixed RCE cell samples was found to be a more reproducible and an easier method to measure the mechanical properties of RCE cells within the resources and time scale available.

2.4.3. Cantilever calibration

Before starting a measurement, the cantilever required calibration to calculate the sensitivity and spring constant of the cantilever as the vertical deflection of the cantilever is usually measured in volts, which can be used for normal AFM modes. However, for modes such as QI™ mode, it is necessary to know the cantilever deflection in units of length or forces. Therefore the volts need to be converted to length/distance or force. This conversion of units was done by cantilever calibration within the JPK SPM software and it is important for measuring the mechanical properties of a sample or the mechanical interaction between the cantilever and sample during imaging.

The JPK SPM software provided several methods of calibration and the standard method had two steps which involved firstly carrying out a force distance curve measurement on a hard substrate such as a glass slide using the force spectroscopy mode and working out the sensitivity fit of the repulsive or linear part of the extend curve. For this measurement a 40 mm petri dish containing PBS was used. Secondly, the cantilever spring constant was determined using the thermal noise method, based on the calculation made by J.L. Hutter and J Bechhoefer. For the calculation of spring constant using this method, the cantilever deflection must be in units of length which is why the sensitivity must be measured before the spring constant. These methods were set up within the JPK SPM software. A correction factor of 0.817 was used due to calibration of the cantilever in liquid as the liquid has a dampening effect on the resonance peaks which affects the thermal noise method and therefore the accuracy of the spring constant measurement. This was a pre-set constant within the software that could be used.

Following cantilever calibration, prepared samples were placed on the AFM ready for measurements in QI™ mode. Images and QI™ data files of 50 x 50 µm areas were obtained and the data count was approximately 16384 per scan with resolution of 128px and each pixel represented a force-distance curve.
2.4.4. QI™ mode – Quantitative Imaging with the JPK NanoWizard® 3 BioScience AFM

AFM technology allows a further insight into the nanometre environment and can be used to measure interaction forces and to determine the mechanical properties of various samples. However, due to the way in which the more traditional imaging modes of AFM work, obtaining this kind of information from more challenging samples such as softer biological samples has its disadvantages. For example in contact mode, high lateral forces could damage or move the sample if a softer biological sample was being measured. Similarly in AC mode the vertical force could lead to softer samples being compressed and therefore damaging the structure. QI™ mode is an imaging mode that does not apply any lateral forces and allows a precisely controlled vertical force at each pixel whilst imaging (JPK Instruments AG, 2011). Therefore this imaging mode was used for all AFM experiments as it allowed both RCE cells and PDMS samples to be measured using standard cantilevers as there were no limitations of sample type, sample geometry or environmental conditions. RCE cell measurements were carried out on single RCE cell and so one RCE cell was measured per scan. The mechanical properties of the single RCE cell were investigated and its mechanical interactions with different substrate properties whilst being attached to that surface. Four PDMS blends were used and measured in triplicate for the PDMS blends without RCE cells attached and for each sample two scans were carried out. For the RCE cell – PDMS samples, each PDMS blend was sampled three times (replicates) and for each sample three RCE cells were measured using QI mode. Four different RCE cell areas were analysed following the collection of data, which provided a sample number of 36 per RCE cell area of interest.

2.4.5. Data processing

The data was processed using the JPK NanoWizard® Data Processing (DP) software (JPK Instruments, Germany). This software enabled the Young’s modulus to be derived from the QI™ force curves obtained, involving several steps. The force curves are shown in Figures 10 and 11, before and after conversion to tip-sample separation for elasticity fit to be applied. All processes would be applied to the approach part of the curve because it would not normally have any other interactions such as adhesion that would make it difficult to identify the contact point. Firstly, the processing options ‘Baseline subtraction’ and ‘contact point determination’ were selected in order to remove any offset or tilt from the curve and to find the contact point. The next step was to select ‘calculate tip-sample separation’, which corrects the height signal for the bending of the cantilever. When force curves are loaded, this function allows plots of force against tip-sample separation rather than plots of force against piezo displacement. After selecting this function, the height signal is automatically corrected for the cantilever bending (difference between cantilever deflection and height, measured) and a
new channel for tip-sample separation is produced. The QI™ data curves are now ready to be fitted with the Hertz model to derive the Young’s modulus, after specifying the tip geometry and half angle as well as Poisson’s ratio (Section 2.4.6) (JPK Instruments, 2015a). This method of data processing was saved and was then applied to all QI™ data files to perform batch processing of multiple force curves using the same setting of parameters.

![Figure 7](image)

Figure 7 – A representative force curve obtained from QI mode imaging scans using AFM. Showing the vertical deflection and height (measured) curve before the elasticity fit procedures are applied.
2.4.6. Hertz Model

The original Hertz model considers the localised stresses between two spherical bodies that come into contact and slightly deform under the imposed loads (Hertz, Jones and Schott, 1896). Equation 7 shows the Hertzian model, where \( F \) is force (from force curve), \( E \) is Young’s modulus, \( v \) is Poisson’s ratio, \( R \) is radius of the indenter or tip and \( \delta \) is the indentation (Johnson, 1987; Rosenbluth, Lam and Fletcher, 2006).

\[
F = \frac{4}{3} \frac{E}{(1 - v^2)} \sqrt{R\delta^3} / 2
\]

Equation 7 - Hertzian Model

The model relates the elastic modulus of a material or cell with the forces and indentation depth measured during a nanoindentation test (Valero et al., 2016). The Hertz model approximates the sample as an isotropic, linear elastic solid that is homogenous. It also assumes that the tip is not deformable so there are no further interactions between the tip and sample. However, biological
samples are described as anisotropic, nonlinear and are not homogenous (Weder et al., 2014). The energy delivered by the indenter or tip is not fully returned by the cell, which would be the case for an absolute elastic material. Instead the energy dissipates due to the plastic behaviour of biological materials, which appears as hysteresis between the extend and retract parts of the force curve (Neumann, 2008). Irrespective of the limitations, the Hertz model is a commonly used method to calculate the mechanical properties of biological samples such as cells (Wenger et al., 2007; Neumann, 2008; Christ et al., 2010; Chopinet et al., 2013; Weder et al., 2014). The nano-scale measurements that are possible using AFM enables this to be a useful tool in determining elastic properties of inhomogeneous biological samples like cells.

One of the values that describe the mechanical response of a material in Hertz Model is Poisson’s ratio and for a soft biological sample, Poisson’s ratio is normally set to 0.5 and assumed to be an incompressible material. However it is observed that biological samples can exhibit a Poisson’s ratio between 0.3 and 0.5 (Jacobs, Huang and Kwon, 2013). The Poisson’s ratio used for this study was 0.5. Different geometries of the indenter or tip other than spherical have different radii of the contact circle and can affect the Young’s modulus calculations. However, several extensions to the original model were made to include different tip geometries (Lin, Dimitriadis and Horkay, 2007). The JPK DP software provided an automatic fitting of the correct indenter shape, in this case for a conical tip, so the user did not have to make an approximation of this, as shown by Equations 8 and 9 (Neumann, 2008).

\[ F = \frac{E}{1-v^2} \frac{2 \tan \alpha}{\pi} \delta^2 \]  
Equation 8 – Hertz model extension for conical tip

\[ \alpha = \frac{2 \tan \alpha}{\pi} \delta \]  
Equation 9 – Hertz model extension for conical tip

where \( \alpha \) = semi-opening angle of the cone
2.4.7. Further analysis following batch processing of QI™ data

Nanoscale measurements were taken of just PDMS samples without the presence of RCE cells on the surface. PDMS 184 (only Sylgard 184) and PDMS blends 10:1, 5:1 and 1:1 (ratio of Sylgard 184: Sylgard 527) were tested using QI™ mode and the data obtained from the scans was used to determine the topography or roughness of the sample surface, the height measured and Young’s modulus of the sample and region of interest. Image files were produced from batch processing, which allowed measurements to be taken of the whole image or 50 x 50 µm scan region, point measurements or histogram data showing average values for a selected area within the processed QI™ data image. The roughness of the PDMS surface was measured using the Setpoint Height channel of the QI™ image data and selecting the histogram tool in the JPK DP software (JPK Instruments, Germany). The roughness data was obtained as an average of a 30 x 30 µm area which included average roughness Ra (arithmetic average or absolute values of the profile height deviations from the mean line), RMS roughness Rq (root mean squared average of the height deviations from the mean line) and peak-to-valley roughness Rt (total height of roughness profile) (Demichelis et al., 2014; Torres, Luis and Puertas, 2015). The average roughness Ra value was used to compare the surface topography of the samples. The representative image area used for PDMS only samples and the measurements carried out is shown in Figure 12.
Figure 9 – JPK DP software (JPK Instruments, Germany) interface showing the roughness data measurements from the Setpoint Height image (30 x 30 \( \mu \text{m} \) area) for PDMS 184 in PBS.

For RCE cell samples, the roughness measurements were taken using the histogram tool but specific areas of the cell were selected to obtain roughness data for the nucleus and cell body of the cell as well as for the substrate the RCE cell was attached too (PDMS or tissue culture plastic). Figure 13 shows a representative image of how the roughness measurements for an RCE cell were taken using the Qi™ data images. This method of highlighting the region of interest on the cell was used across all images for RCE cell measurement of surface roughness and was also used to measure the roughness of the cell body as well as the surrounding PDMS or tissue culture plastic.
To further analyse the QI™ data obtained following batch processing and Young’s modulus calculations, images for the setpoint height and Young’s modulus were used to extract data for specific areas of the RCE cell or PDMS sample. This was done using the point measurement tool in the JPK DP software (JPK Instruments, Germany) and by using the height image alongside the Young’s modulus processed image (Figure 14), the data could be extracted simultaneously and at the correct region of interest on the RCE cell. This method was used for all processed QI™ data and applied to both RCE cell samples and PDMS samples.
Figure 11 - JPK DP software (JPK Instruments, Germany) interface showing how the height and Young’s modulus point measurements were carried out for RCE cell samples and just PDMS sample. Figure A shows the setpoint height image of an RCE cell attached to PDMS and figure B shows the analysed Young’s modulus data image corresponding the figure A. Both A and B show how the point measurement tool was used to extract the analysed data from specific areas of the RCE cell. Figures C and D show the same process but for a PDMS sample without RCE cell attached.
2.5. Rabbit Corneal Epithelial Cell Culture

The rabbit corneal epithelial (RCE) Cell Line was procured from the European Collection of Authenticated Cell Cultures (ECACC), Public Health England. Cultures were started at passage 8 and a working bank of passage 11 RCE cells were used in experiments going forward. The immortalised RCE cell line was established following infection of primary cultured rabbit (New Zealand Albino) corneal epithelial cells with a recombinant SV40 vector and was therefore classed as a genetically modified organism class 1 (GMO1). This allowed continuous culture of the corneal epithelial cells and a continued proliferation and growth over several generations and passages without phenotypic changes (Araki et al., 1993). The RCE cell line provided a research tool that was used to establish protocols with an easy to handle cell line in vitro without the setbacks of primary corneal cell culture such as a lower cell yield, heterogeneity in culture, and a poor proliferative ability and therefore short life span. A rabbit cell line was used as a proof of concept cell line for process development. It has also been shown in the literature that the rabbit ocular surface and rabbit cell lines has been used for several years as a reliable comparison to human corneal injuries (McIntosh et al., 2013; Thomasy et al., 2014).

2.5.1. RCE cell culture medium formulation

RCE cells were cultured in Dulbecco’s modified eagle medium (DMEM): Ham’s F12 medium (1:1 ratio) with 15 mM hepes and L-glutamine (Lonza, distributed through Scientific Laboratory Supplies (SLS), UK). To this basal medium, the following reagents were added; 5 µg/ml insulin solution human (Sigma-Aldrich, UK), 10 ng/ml epidermal growth factor (EGF) mouse recombinant (Life Technologies, UK), 0.1 µg/ml cholera toxin from *Vibrio cholerae* (Sigma-Aldrich, UK), 0.5 % dimethyl sulphoxide (DMSO) (Sigma-Aldrich, UK) and 15 % foetal bovine serum (FBS), heat inactivated, South American origin (SLS, UK and Life Technologies UK). After using this culture medium formulation with the RCE cells, it became apparent that the FBS could be reduced to 10 % FBS and this was the concentration of FBS used in the RCE cell culture medium for all experiments using PDMS and cell culture going forward. RCE cells were cultured in an incubator at 37 °C and 5 % CO₂ using Nunc T75 tissue culture flasks and Nunc 6-well tissue culture plates. The volume of completed culture medium (basal DMEM:F12 plus supplements) required in a T75 flask was 15 ml and in one well of a 6-well plate 2 ml was used.

2.5.2. Thawing of cryopreserved RCE cells

Rabbit corneal epithelial (RCE) cells were obtained as a frozen vial of cells at passage 8, procured from the European Collection of Cell Cultures (ECACC), Public Health England (RCE (ECACC
On receipt of delivery the vial was immediately stored in liquid nitrogen. After cell resuscitation from cryostorage, the cell suspension was then centrifuged at 200 g for 5 minutes and after this the supernatant containing freeze medium was removed taking care not to dislodge the cell pellet. The cell pellet was re-suspended in the correct volume of complete medium following viable cell count calculations per ml. This ensured there was a known concentration of cells per ml of cell suspension.

The recommended seeding density was 2-4 x10, 000 cells/cm² for the RCE cell line (European Collection of Cell Cultures (ECACC), Public Health England). However after culturing the RCE cells over several passages, it was found that the cell seeding density in a T75 flask for optimum cell growth and viability was approximately 5.3 x 10³ cells/cm², which grew to 70-80 % confluent over 2 days of cell culture. Decreasing the seeding density increased the time it took for the cells to grow to a sub-confluent layer so cells were in culture longer before they were passaged. The seeding density could be adjusted dependant on the number of cells required for a particular experiment. Cell culture flasks were prepared in the BSC and the correct volume of fresh complete medium was added to each flask. The flasks were incubated at 37 °C, 5 % CO₂ and 95 % humidity until cells were sub-confluent (70-80 %). To remove any remaining freeze medium and dead cells, a medium change was performed in between cell passages. Spent medium was removed according to correct disposal methods and fresh pre-warmed complete medium was added to the flask.

2.5.3. Passaging RCE cells

When the RCE cells had become sub-confluent (70-80 % confluent), the flask was removed from the incubator and placed in the BSC. The spent medium in the flask was removed and the cells were then washed with 15 ml of phosphate buffered saline (PBS) (1X, without Ca and Mg, Lonza). To detach the cells from the T flask, 3 ml of trypsin/EDTA (pre-warmed in a water bath at 37 °C for 30 minutes) was added to cover the cells and the flask was placed in an incubator at 37 °C, 5 % CO₂, 95 % humidity for 5 minutes. 7 ml of pre-warmed complete medium was pipetted into the T flask and the cell suspension was centrifuged at 200 g for 5 minutes. The resulting cell pellet was resuspended in the correct volume of complete RCE cell culture medium, calculated from the cell count to provide the required concentration of cells per ml, which was 5 X 10⁵ or 1 X 10⁶ cells per T75 flask. The total working volume of the flask was 15 ml so complete RCE cell medium was made up to 15 ml with the volume of RCE cell suspension required.
2.6. RCE Cell Culture on PDMS Blends

The sterilised PDMS samples were placed into 6-well plates, one PDMS sample per well with the plasma treated side facing upwards. This was recognised by the water droplets on the PDMS sample as it was removed from the container and it was clearly visible which side had been plasma treated due to larger areas of the treated surface being more hydrophilic compared to the untreated surface. These had already been cut to size before plasma treatment and autoclaving, as described in Section 2.1.3, Chapter 2.

After cell counting, the correct seeding density required for the experiment was calculated and the RCE cell pellet was re-suspended in the correct volume of complete RCE cell culture medium. 2 ml of complete RCE cell culture medium was added to each well containing PDMS to cover the base of the well and the PDMS. The correct volume of RCE cell suspension was then added to each well to seed the PDMS surface. The seeding density used for these experiments was $8.3 \times 10^3$ cells/cm². The 6-well plates were kept in an incubator at 37 °C, 5 % CO₂, 95 % humidity. After 24 hours in culture, the PDMS–RCE cell samples were used for further experiments. In order to ensure that the RCE cells attached to the PDMS samples, cell culture inserts were used (Sigma – Aldrich, UK) to hold the RCE cell suspension over the PDMS samples in the well.

2.6.1. Fixation of RCE cell–PDMS samples

Once RCE cell–PDMS samples were prepared and after they had been cultured for 24 hours, the RCE cells were fixed using formaldehyde solution (Sigma-Aldrich, UK). The formaldehyde was procured at a concentration of 37 % and was diluted using a 1 in 10 dilution to produce a 3.7 % solution using sterile PBS. Cell culture medium was aspirated from each well and the RCE cell–PDMS samples were washed once with PBS. The diluted formaldehyde solution was pipetted into each well carefully so as not to disrupt the RCE cell–PDMS sample and 3 ml was added to each well to fix the RCE cells cultured on PDMS for 30 minutes at room temperature. After the fixation period, the formaldehyde solution was aspirated and each well was washed three times with PBS to remove the fixation solution. Samples were then stored in PBS at 4 °C until required.

2.6.2. Attachment of RCE cells to PDMS after 24 hours in culture using Actin and nuclei staining of fixed RCE cell–PDMS samples

Actin filament and nuclei staining were carried out using Alexa Fluor® 568 phalloidin stain (Molecular probes by Life Technologies, Thermo Fisher Scientific, UK) and 4’, 6–diamidino–2–phenylindole, dihydrochloride (DAPI) stain (Molecular probes by Life Technologies, Thermo Fisher Scientific, UK). Alexa Fluor® 568 phalloidin is a high affinity probe for filamentous actin (F-actin) and is conjugated
to the Alexa Fluor® 568 red fluorescent dye (Excitation/Emission 578/600 nm). F-actin is one of the major components of the cytoskeleton and plays an important role in several dynamic cell processes (Lee and Dominguez, 2010). DAPI is a nuclear and chromosome counterstain and once it is bound to AT regions of DNA, emits blue fluorescence (Excitation/Emission 350/470 nm), to visualise the nuclei clearly against other contrasting fluorescent dyes for other cell structures of interest. The PBS covering the RCE cell–PDMS samples was aspirated off each well and before the staining procedure samples were carefully transferred to new 6–well plates using sterile tweezers. This step was done to avoid any staining of RCE cells that had attached to the base of the tissue culture plastic plate.

Intracellular (IC) permeabilisation buffer (Thermo Fisher Scientific, UK) was used before staining and was diluted to a 1X concentration in PBS. This buffer contained PBS pH 7.3, fetal calf serum, saponin and 0.1 % sodium azide and was used for permeabilising cells for intracellular staining and surface antibody staining. 2 ml of the diluted solution was added to each well to cover the samples and was left for 5 minutes at room temperature. The permeabilisation buffer was aspirated off and each sample was washed twice with PBS. A further 2 ml PBS was added to each well and to this 6 µl of the Alexa Fluor® 568 phalloidin stain was added directly to each well. The plate was rocked gently back and forth to mix the phalloidin stain with the PBS. Plates were covered with foil to minimise the exposure of fluorescently labelled samples to light. Foil was used to reduce potential photo bleaching of the dye, which could affect the fluorescence microscopy measurements. Samples were kept at room temperature for 1 hour to incubate with the phalloidin stain.

After this the PBS and phalloidin stain was aspirated and samples were washed three times with PBS to remove any excess dye that had not bound to the RCE cells. A working solution of DAPI at a concentration of 300 nM was made up in PBS (1 µl stock solution in 10 ml PBS) and 2 ml of this solution was added to each sample, the plate was wrapped in foil and incubated at 37 °C for 15 minutes. Following this incubation period, the DAPI staining solution was aspirated and samples were washed three times with PBS to remove excess unbound stain. The samples were kept in PBS and the plates were wrapped in foil and stored at 4 °C until required for fluorescent microscopy. Fluorescent images were taken at 20x magnification on untreated and plasma treated RCE cell–PDMS samples using the Nikon Eclipse Ti microscope and digital camera. For this study, the positive control was RCE cells cultured on tissue culture plastic and the negative control was PDMS sample cultured without RCE cells.
2.6.3. Quantification of RCE cell attachment to PDMS blends using the Nikon BioStation CT

Fixed RCE cell–PDMS samples (method detailed in Section 2.6.1) were used to quantify RCE cell attachment after 24 hours in culture and area coverage and spread of RCE cells across each surface of untreated and plasma treated PDMS blends. This was carried out using the Nikon BioStation CT, an automated imaging and incubator system. The following method was carried out within a BSC using sterile reagents.

After fixation, the methods used to stain RCE cell–PDMS samples with phalloidin and DAPI described in Section 2.6.2 were followed until the last step of counterstaining the nuclei with DAPI. Instead, a red nuclear stain was used as the BioStation CT did not have a blue fluorescence imaging channel and could not detect a blue fluorescent stain. The red nuclear stain was used to enhance the fluorescent signal rather than as a counter stain, enabling greater detection of RCE cells through the automated imaging system within the BioStation CT. After the RCE cell–PDMS samples had been stained with phalloidin, a working solution of ethidium homodimer–1 (EthD-1) was prepared by diluting the stock solution 10 µl in 10 ml PBS. This was made up according to the volume required and 3 ml of the EthD-1 working solution was added to each well to cover each sample. The plate was wrapped in foil and incubated for 37 °C for 15 minutes. After this the samples were washed twice in PBS and an additional 2 ml PBS was added to each sample before the plate was placed in the Nikon BioStation CT.

Once the plate had been placed within the carrier, the process was automated beyond this point by using the touch screen user interface to set imaging experiments. Imaging parameters used were full well scans of each sample at 10x magnification taking images in phase contrast (Ph) and the red fluorescent channel (Ch3). Both phase contrast and fluorescent images were taken to ensure the analysis software (Nikon CL-Quant) was able to correctly count RCE cells attached to the PDMS surface. Fluorescent images were used for image quantification and automated RCE cell count parameters were checked against phase contrast images to ensure only areas of RCE cell coverage were being measured by the software. Tiling images were taken across the whole well using grid sizes of 7 x 7 images, which were stitched together (overlap of 50 pixels) in the automated image analysis software Nikon CL-Quant as shown in Figure 15. Further image analysis was carried out using the Nikon CL-Quant automated image analysis software using preconfigured image analysis assays and batch processing. The method of analysis used was a set of procedures customised to the RCE cell–PDMS sample images to ensure the correct measurement of RCE cells on the surface of the PDMS blends. The percentage coverage of RCE cells was calculated by dividing the total count (total
masked area which was the RCE cells as selected by the image analysis software) by the image area and multiplying by 100.

Figure 12 – Representative images showing how the 7 x 7 tiling images taken of the whole well containing the fluorescently stained RCE cell – PDMS samples in the Nikon BioStation CT were stitched together. The stitch overlap applied was 50 pixels. Scale bar shows 800 µm. This was done using the Nikon CL-Quant analysis software to see the attachment of RCE cells over the whole sample surface. Image A shows the brightfield images of the whole well with the fluorescence overlap and image B shows the fluorescence channel 3 images of the whole well.
2.6.4. PrestoBlue® Cell Viability Assay

PDMS samples were seeded at 8.3 x 10^3 cells/cm². The plate was incubated for 24 hours at 37 °C 5 % CO₂ and following this period the PrestoBlue® viability assay (Molecular probes by Life Technologies, Thermo Fisher Scientific, UK) was used to quantify cell viability. The PrestoBlue® reagent was used as a live cell assay as it does not require cell lysis and allowed the quantitative non-destructive analysis of cell viability and proliferation of the RCE cells over time when cultured on the different PDMS substrates. Within the PrestoBlue® reagent, Resazurin (blue, non-fluorescent) is reduced by metabolically active cells and changes to Resorufin (red, fluorescent). The colour change can be detected by fluorescence or absorbance optical intensity (Excitation/Emission 544/590 nm).

For the PrestoBlue® assay, a 1 in 10 dilution of the PrestoBlue® reagent to cell culture medium was required. After the first 24 hours in culture and before the PrestoBlue® experiment for day 1 had begun, RCE cell–PDMS samples were transferred to a new 6–well plate using sterile tweezers in order to only measure the viability of RCE cells attached to the PDMS surface for the duration of the assay. The 24 hour spent RCE cell medium was kept and 200 µl of the 24 hour RCE cell culture medium was removed from each well (2 ml total starting volume). 200 µl PrestoBlue® was added to the remaining medium. The plate was rocked gently to ensure that the PrestoBlue® reagent had mixed with the RCE cell culture medium and the plate was wrapped in foil and incubated for 40 minutes at 37 °C 5 % CO₂. Following incubation, the medium and reagent were gently swirled in the plate and three 100 µl samples of the medium with PrestoBlue® were taken and added to a black 96–well plate with a clear bottom. As the plate containing the RCE cell–PDMS samples was to be continued for 7 more days, the experiment was carried out within a BSC to ensure sterility of the sample.

The black, clear bottom 96–well plate containing the PrestoBlue® medium samples was placed in the FLUOstar Omega plate reader to measure the fluorescence following sample incubation with PrestoBlue® at 544 nm excitation and 590 nm emission wavelengths. A positive control of 100 % reduced PrestoBlue® reagent (done by autoclaving a 1 in 10 concentration of PrestoBlue® reagent in RCE cell culture medium at 121 °C for 15 minutes) and a negative control (only RCE cell culture medium) were also read at each time point during the 8 day experiment. After each reading, the medium containing PrestoBlue® was aspirated, each well containing RCE cell–PDMS samples was washed once with PBS, and 2 ml of pre-warmed complete RCE cell culture medium was added to each well and the plate was kept in an incubator until the next reading. This method was repeated on the same RCE cell–PDMS samples until day 8 and after this time point was measured, samples were discarded.
2.7. RCE Cell Migration Measured by Scratch Wound Assay Using the Nikon BioStation CT

The motility and collective migration of RCE cells cultured on the surface of the PDMS blends was investigated using the Nikon BioStation CT for automated imaging at set points of interest and images were taken at particular time intervals. Images were taken in phase contrast at 10x magnification for all conditions. The scratch wound assay was used to study cell migration of RCE cells and was carried out by manually creating a “scratch” in a confluent monolayer of cells (Liang, Park and Guan, 2007). This assay was used to determine the rate of cell migration of the RCE cells cultured on both tissue culture plastic and the different PDMS blends described previously in Section 2.1.3 of this Chapter. The effects of PDMS with different material properties on RCE cell migration were observed in comparison to RCE cell migration on tissue culture plastic.

2.7.1. RCE cell migration assay when cultured on tissue culture plastic

To begin with, a gel pack was submerged in a water bath at 37 °C, which was done to reduce the condensation on the plates due to transport from room temperature to 37 °C in the BioStation CT incubator. 2 ml of pre-warmed complete RCE cell culture medium (see Section 2.5.1) was added to each well. Once the RCE cells were passaged from tissue culture flasks, the RCE cell suspension was seeded into the 6-well plate at a concentration of 3.96 x 10^4 cells/cm^2. A higher cell concentration was used so that a confluent layer of RCE cells was formed after 24 hours in culture, reducing the culture period before the start of the scratch wound assay. This particular seeding density was used because it was calculated as the correct concentration of RCE cells to form a confluent monolayer of RCE cells once attached to the surface in 24 hours. This also limited the proliferation of RCE cells prior to performing the scratch wound assay so that what was measured using the assay was cell motility. The plate was incubated at 37 °C 5 % CO_2 for 24 hours for the RCE cells to attach and form a confluent monolayer of cells covering the base of the well. After this incubation period, the scratch wound assay was started by physically making a gap or “scratch wound” in the confluent RCE cell layer using a sterile pipette tip. 20–300 µl size sterile pipette tips were used. To do this, the plate was removed from the incubator and placed in a BSC. The spent medium in each well was removed and the RCE cells were washed with 2 ml sterile PBS to remove any residual spent medium. An additional 2 ml of PBS was added to each well and while the RCE cell layer was covered by PBS, using a sterile pipette tip, a scratch through the cell layer along the centre of the well was created. This was done by manually moving the pipette tip in one continuous movement from top to bottom in the well, along the vertical axis, ensuring to scrape away the RCE cell layer under the pipette tip while doing so. The pipette tip was discarded and a new sterile tip was used for the next well.
Following this the PBS was aspirated away to remove the RCE cells that had been scraped away from the plate in the scratch wound and 2 ml pre-warmed complete RCE cell culture medium was added to each well. This method is a simple, cost effective way to perform a cell migration assay when compared to other methods such as the Boyden chamber assay, microfluidics–based assays and barrier assays that are also available (Liang, Park and Guan, 2007; Grada et al., 2017).

The plate was then transferred to the Nikon BioStation CT after placing in the specified plate holder for the BioStation. The transfer from the BSC to the incubator environment within the BioStation produces condensation inside the plate so first a pre-warmed sterile 6-well plate was used to change the lid on the 6-well plate containing the RCE cell–PDMS samples to help reduce condensation. Then the pre-warmed gel pack at 37 °C was placed on the surface of the plate in the plate holder and carried carefully to the BioStation and removed to place the plate into the carrier in the BioStation. This was done to try to reduce some of the condensation by keeping the plate warmer than room temperature. Images were taken along the “scratch wound” edge and these imaging points were set as custom points to be imaged every 30 minutes at 10x magnification.

The time-lapse images collected along the “scratch wound” edge were analysed using the Nikon CL-Quant analysis software. A standard wound healing recipe pre-set in the analysis software was modified to the RCE cell images to accurately measure the gap closure in the scratch wound assay. The masking used by the analysis software is shown in Figure 16. This showed how the scratch wound was selected for and measured across all images taken of the scratch wound assays over time. Figure 16 also shows that the software was able to select the gap in the RCE cells or the wound only because the wound healing recipe used to analyse the images was optimised to the RCE cells by using an image to modify the parameters such as the threshold for the masking.

2.7.2. RCE cell migration assay when cultured on PDMS blends
PDMS blends were prepared as previously described. Prior to seeding, 2 ml of pre-warmed complete RCE cell culture medium (see Section 2.5.1) was added to each well containing PDMS. For the scratch wound assay on PDMS, 3.96 x 10⁴ cells/cm² RCE cells were added to the medium per well in a 6–well plate containing PDMS samples. The plate was kept in an incubator at 37 °C 5 % CO₂ for 24 hours so that the RCE cells could attach to the PDMS and form a confluent cell layer over the surface.

After observing a confluent RCE cell layer over the surface of the PDMS samples, the scratch wound assay was performed as described previously in Section 2.7.1 of this Chapter. The same method to produce the “scratch wound” was used on RCE cells cultured on tissue culture plastic and on PDMS
blends. These samples were also measured using the Nikon BioStation CT using the same imaging parameters and pre-set analysis as those used for tissue culture treated plastic.

The percentage wound closure was calculated by using the wound area to size ratio obtained when the modified wound healing recipe was applied and this value was multiplied by 100. This was then used for the 0-12 hour period compared across all samples and the change in wound size between each image was calculated to get the change over time. Also this method of image analysis allowed the optimisation of wound widths so that at the start of the assay, similar sized wound widths were created.
Figure 13 – Representative images showing the method of analysis used for the scratch wound assay in the BioStation CT. The images in A and C show the RCE cells attached to TCP at different time points without the analysis. Images in B and D show the same images as A and C with the analysis recipe applied and the masking can be seen. This will give the area of the wound at the different time points during the assay and other measurements such as the whole image area and the ratio of the wound to cells. The percentage wound closure was calculated by using the wound size divided by the whole image area.

2.8. Statistical analysis

All statistical tests were carried out using Minitab® 17 and data was analysed using analysis of variance (ANOVA) tests, with Tukey analysis, to determine whether there were differences between the PDMS blends being tested under specific experimental conditions. ANOVA uses variances to determine whether the means of two or more populations are different (Minitab® 17 Statistics
Any statistically significant differences between PDMS blends were identified following statistical analysis and by using a multiple comparison method called Tukey’s to obtain more information about the differences between specific means (see results Chapters 3-5 for statistical analysis). Another type of statistical analysis carried out was a regression analysis, shown in Figure 42, Chapter 4. Regression analysis can be used to investigate the statistical relationship between one or more predictors and the response variable. New observations can then be predicted using the equation generated in Minitab® (Minitab® 17 Statistics Guide). The data in Figure 42, Chapter 4 shows the predicted seeding density or cell number estimated from a regression analysis carried out on data obtained from a PrestoBlue calibration curve (for PrestoBlue methodology see Section 2.6.4). This was done using different RCE cell seeding densities and relative fluorescence values for each density (minus the cell culture medium only control fluorescence value) were used in the regression analysis. The fit regression model tool in Minitab® 17 was used to model the relationship between the cell seeding density and the fluorescence values obtained. This generated a fitted line plot for the quadratic model applied, which showed that the relationship between seeding density and fluorescence minus control was statistically significant (P < 0.001). Therefore the model could be used to predict the seeding density or cell number of a particular fluorescence value obtained in the experiments carried out in Figure 41, Chapter 4. Minitab® 17 generated a prediction report and a model report which was used to estimate seeding densities.

### 2.9. Summary

In summary, this Chapter details the methodology used to carry out the experimental work described in Chapters 3–7 and the analysis methods used on the data collected. Further discussion of the results obtained is carried out in each results Chapter, followed by an overall discussion and conclusions Chapter (Chapter 7). More details on each experimental design and parameters used and how the methods discussed in this Chapter applied to the experiment are given in Chapters 3-7.
Chapter 3
Modification of Surface Chemistry of Polydimethylsiloxane and Testing of the Bulk Mechanical Properties for Cell Culture
3.1. Introduction

Polydimethylsiloxane (PDMS) is a silicone elastomer and has many desirable properties that make it a suitable substrate for biomedical applications. It is a biocompatible, thermally stable, durable and transparent polymer that is simple to handle and cost-effective (Mata, Fleischman and Roy, 2005; Sugiura et al., 2008). Also, due to its ability to easily mold into sub-micrometre features, ease of bonding to itself and glass, high chemical resistivity and gas permeability, PDMS is most commonly used for the fabrication of microfluidic devices (Zhou et al., 2012). PDMS has been increasingly used for soft lithography techniques such as micro-contact printing, replica molding and micro-molding in capillaries (Mata, Fleischman and Roy, 2005). It also been used in biomedical applications such as a biomaterial in catheters, drainage tubing, insulation for pacemakers, membrane oxygenators and ear and nose implants (Mata, Fleischman and Roy, 2005; Pinto et al., 2010). PDMS has also been used for soft contact lenses due to its high oxygen permeability. However, PDMS requires further modifications for most biological applications as PDMS is a hydrophobic material (Liu and Sheardown, 2005; Farandos et al., 2015).

In the field of tissue engineering, there is an increasing need for culture substrates and materials that are well-defined in terms of their mechanical and surface properties and this will impact on the success of tissue constructs and key factors in producing these constructs such as stem cell differentiation (Wong, Leach and Brown, 2004; Jones, Hamley and Connon, 2012). PDMS and other substrates have been used previously to investigate the responses of cells to changes in the substrate mechanical properties and surface properties. Cell and tissue scaffolds have been produced from ECM such as collagen, gelatin and fibronectin and have also been developed from other polymers such as PVA and PET, using a number of different techniques such as electrospinning (Ma et al., 2005; Karimi and Navidbakhsh, 2014).

PDMS was chosen due to its many advantageous properties and the ability to change the bulk mechanical properties by using different types of commercially available Sylgard®. The flexibility of the polymer meant that several different mechanical properties could be investigated and using different mixing ratios could create a range of PDMS substrates with different elastic moduli. This allowed the preparation of a 2D substrate for cell culture using a material that could be modified in terms of surface and bulk mechanical properties to study the effects of these changes on rabbit corneal epithelial (RCE) cells.

This Chapter discusses the results from the PDMS surface modification used to produce a more hydrophilic surface to promote cell adhesion. Differences in the bulk mechanical properties are also
discussed; in particular, the Young’s modulus of the PDMS blends produced and the factors affecting this in terms of the testing methods used and process parameters.

### 3.1.1. Aims and Objectives

The aim of this Chapter is to investigate the surface and bulk mechanical properties of the PDMS blends used in experimental work going forward. The objectives to achieve the aim are to test the effects of surface modification on the PDMS wettability and surface chemistry and to investigate whether the changes to the surface will produce a suitable surface for cell adhesion. Another objective is the mechanical testing of PDMS substrates to further understand the bulk mechanical properties of the PDMS blends using three different measurement techniques. Following this the range of elastic moduli can be determined in relation to the differing PDMS blends and how these differences in bulk mechanical properties affect cell adhesion and cell behaviours.

#### 3.1.1.1. Hypothesis

It is hypothesised that following surface modification of the PDMS blends, the surface will become hydrophilic and wettability measurements will confirm that the modified PDMS surfaces can promote cell adhesion. Following the mechanical testing of the PDMS blends, it is hypothesised that with increasing ratios of Sylgard® 527 compared to Sylgard® 184, the elastic modulus of the PDMS blends will decrease. Therefore from PDMS 184 (pure Sylgard® 184) to PDMS 10:1 (Sylgard® 184: Sylgard® 527), PDMS 5:1, PDMS 1:1 and PDMS 1:5 a decreasing trend should be observed in the mechanical properties of the blends.
3.2. Results

3.2.1. Cold atmospheric pressure plasma treatment of the PDMS surface

3.2.1.1. Wettability of the surface of PDMS on untreated and plasma treated samples
Due to the hydrophobic nature of the polydimethylsiloxane (PDMS) surface, a cold atmospheric pressure (CAP) dielectric barrier discharge plasma jet was used to plasma treat the surface of the PDMS to produce a more favourable surface for cell attachment and further biological work. This method of surface modification has been described in more detail in Chapter 2, Section 2.2.

It was found that following plasma treatment of PDMS, the water contact angle (WCA) decreased from 107.6 °±9.0 ° to 31.1 °±8.4 ° and furthermore there was a statistically significant decrease in the WCA following plasma treatment (Figure 17). The representative images in Figure 18 showed the profile of a water droplet on an untreated PDMS surface (a) and plasma treated surface (b). This also demonstrated a clear change in WCA between untreated PDMS and plasma treated PDMS. After plasma treatment, a lower WCA was observed as supported by Figure 18b showing the spread of the water droplet on the plasma treated PDMS surface. This highlighted the change in the hydrophobicity of PDMS following plasma treatment and the surface became more hydrophilic.
Figure 14 - Water contact angle measurements of untreated and plasma treated PDMS 184 (mean ± SD, n=4). *** shows a statistically significant difference decrease in contact angle on plasma treated PDMS samples, \( p \leq 0.001 \) (one-way ANOVA using Tukey analysis, Minitab).

The oxygen plasma used generated reactive oxygen species that modified the surface chemistry of the PDMS. Oxygen plasma has been widely used to modify the surface of PDMS to reduce the hydrophobicity and it has been reported that the plasma treatment causes chemical changes in near surface region of PDMS. Hydroxyl groups (Si-OH groups) that are polar functional groups are
introduced into the PDMS surface, producing a more hydrophilic surface (Bodas, Rauch and Khan-Malek, 2008) as shown in Figure 19, a representative diagram of the oxidised PDMS surface. The changes in surface properties of PDMS after oxygen plasma treatment were further investigated in this Chapter using X-ray photoelectron spectroscopy (XPS) and also in Chapter 5 where atomic force microscopy and further surface characterisation is carried out.

![Figure 16 - A representative diagram showing the Si-OH groups or silanol groups attached to the surface after oxygen plasma treatment (adapted from Li, Lei, Sheadel, Xu, & Xue, 2012).](image)

It was clear that the plasma treatment reduced the hydrophobicity of the PDMS surface. However the effects were not permanent, which was observed when plasma treated PDMS was left exposed to the air. This has also been observed by various groups that the surface properties of the PDMS steadily change during ageing and the surface will eventually recover its hydrophobicity after a short duration post plasma treatment (Murakami, Kuroda and Osawa, 1998; Bodas and Khan-Malek, 2007; Zhou et al., 2012). The reduction of the plasma treatment effect was further investigated to understand the time limitation on maintaining the treatment effects for further sample processing, here autoclaving for the sake of sterilisation. This was studied by measuring the WCA over a period of three hours to investigate the change in WCA over time with/without autoclaving (Figure 20).
Figure 17 – Hydrophobic recovery of PDMS 184 over a period of 3 hours after autoclaving. Zero time point represents immediately after autoclaving. Samples that were not autoclaved and plasma treated were also measured immediately after plasma treatment (time point 0). Measurements carried out on dry samples kept in petri dishes (mean value for each time point ± SD, n=3).

It was found that over a period of 3 hours the WCA increased significantly for the plasma treated PDMS samples in the non-autoclaved condition and greater hydrophobic recovery of the PDMS surface was observed (Figure 20). It was observed that without plasma treatment, the autoclaving step in the process did not significantly affect the wettability of the surface of PDMS 184. Both the untreated samples with and without autoclaving showed no dramatic change in the WCA when comparing the measurements taken at 0 and 3 hours. The PDMS samples that had been plasma treated and autoclaved had an overall higher WCA when compared to just plasma treated PDMS, but the WCA values were more similar to each other by 3 hours. Moreover there is a clear difference in the trend of WCA, considering the autoclave effects. A more stable sample was produced by autoclaving the plasma treated PDMS as evidenced by the small change of WCA with time when compared to the plasma treated PDMS that was not autoclaved, which showed a dramatic increase of WCA with time. The plasma treated and autoclaved condition showed a 4.2 % reduction in WCA when comparing time point 0 (93 ° ± 5.7 °) to 3 hours (89.1 ° ± 8.7 °). However, the change of WCA of the un-autoclaved plasma treated PDMS showed a 48 % increase when time points 0
(39.3° ± 14.3°) and 3 hours (82.8° ± 8.4°) were compared. These results suggested a much less stable PDMS surface in the plasma treated condition without autoclaving when exposed to the environment but further supported the improved stability of the WCA on the plasma treated PDMS surface following autoclaving.

The hydrophobic recovery of the PDMS surface occurs within a few hours because of the migration of uncured oligomers from the bulk PDMS to the surface and is a common problem with plasma surface treatments in that the surface modifications are not permanent (Lee and Yang, 2012; Zhou et al., 2012). The oxygen plasma treated PDMS samples were stored in distilled water following treatment. The likelihood of the PDMS samples swelling in the distilled water was not investigated however it is known that using organic solvents with PDMS can cause it to swell (Zhou et al., 2012; Johnston et al., 2014). Although the surfaces of the PDMS samples were made more hydrophilic, it has been shown that storing PDMS under water following oxygen plasma treatment can slow down the rate of hydrophobic recovery (Lee and Yang, 2012). The methods used for this work were to store the plasma treated PDMS in distilled water and further processing was carried out in water too (see Chapter 2, Section 2.2.1).

3.2.1.2. Surface chemistry of untreated and plasma treated PDMS
The changes to the surface of PDMS following plasma treatment were further investigated using X-ray photoelectron spectroscopy (XPS) to look at the changes in the surface elemental and chemical composition after plasma treatment. This was carried out on all blends of PDMS produced, which is described in more detail in Chapter 2, Section 2.2.3 and also the preparation of PDMS blends is detailed in Chapter 2, Section 2.1. The blend ratio of 1:10 (Sylgard® 184: Sylgard® 527) was not continued on from preliminary tests as it was found to be too soft to be handled with tweezers and therefore was a more challenging material to work with for the plasma treatment, sterilisation and further experiments. Mechanical testing was attempted on this PDMS blend but failed due to the loss of the integrity of the bulk material during testing and therefore was deemed unusable for future experiments. The plasma treatment used was cold atmospheric pressure (CAP) plasma (described in more detail in Chapter 2, Section 2.2) using a gas flow through the plasma jet of 3 standard litres per minute of helium with a 0.5 % admixture of oxygen.
Figure 18 – X-ray photoelectron spectroscopy showing the atomic percentage profile of untreated and plasma treated PDMS blends. Survey scans carried out on each blend of PDMS before and after plasma treatment, high resolution scans for Silicon, Oxygen and Carbon (mean ± SD, n=3). *** showed statistically significant increase in oxygen and a statistically significant decrease in carbon following plasma treatment across all blends. For all statistically significant differences p values ≤ 0.001 (One-way ANOVA using Tukey analysis, Minitab).

The results showed a significant percentage change in carbon and oxygen atoms present on the surface of the PDMS blends after plasma treatment. There was a statistically significant decrease in the atomic percentage of carbon present on the PDMS surface across all blends as well as a significant increase in the atomic percentage of oxygen present on the PDMS surfaces following plasma treatment (Figure 21). The silicon present on the surface showed no significant differences before or after plasma treatment (Figure 21).

However, it was also observed that on the surface of one of the PDMS blends 1:5 (Sylgard 184:Sylgard 527), after plasma treatment, a significantly higher atomic percentage of carbon was still present when compared to PDMS 184, 10:1 and 5:1 PDMS blends. A significantly lower atomic percentage of oxygen was also detected after plasma treatment on PDMS blend 1:5.
when compared to PDMS 184 and PDMS blend 10:1. However the atomic percentage of oxygen on the PDMS 1:5 surface following plasma treatment was not significantly different when compared to PDMS blends 5:1 and 1:1. These results suggested that the difference in the surface material properties of PDMS 1:5 that were observed prior to plasma treatment did not affect the outcome of the plasma treatment.

It was expected that a reactive oxygen species would be generated within the plasma jet, which related to the observed increase in the atomic percentage of oxygen on the PDMS surface following plasma treatment (Liu et al., 2010). During plasma treatment radicals are produced in the polymer chains that are found at the surface and in order to do this hydrogen atoms are removed. Some of radicals generated react with the plasma and form functional groups on the surface of the material being plasma treated (Bodas and Khan-Malek, 2007; Pinto et al., 2010). The reaction with oxygen plasma can produce SiO$_2$, Si-OH, or Si-CH$_2$OH groups on the PDMS surface (Sharma et al., 2007; Pinto et al., 2010). The decrease in water contact angle after oxygen plasma as shown in Figures 17 and 18 is due to the presence of hydrophilic silanol groups (Si-OH) and the removal of hydrophobic methyl groups (Si-CH$_3$) from the surface of PDMS due to the plasma treatment, producing a more hydrophilic surface (McDonald et al., 2000; Fuard et al., 2008). The presence of hydroxyl groups are known to be responsible for decreasing the hydrophobicity of the PDMS (Bodas, Rauch and Khan-Malek, 2008). The attachment of these functional groups to the surface of the PDMS following oxygen plasma treatment was detected by XPS and this correlated to the increase in oxygen in Figure 21.

As the oxygen was bound to the PDMS surface and the polymer chains, the atomic percentage of silicon present at the surface did not change drastically (Mata, Fleischman and Roy, 2005). The fall in carbon observed when untreated and plasma treated PDMS samples were compared correlated with the rise in oxygen following plasma treatment and this has been reported as an effect of oxygen plasma in the literature (Kim, Kim and Jeong, 2011). Oxygen plasma treatment is widely used for sterilisation of surfaces in various applications in healthcare and in the food industry (Kong et al., 2009; Pankaj et al., 2014). Plasma is an effective method in breaking organic bonds (e.g. C-H, C-C, C=C) of carbon contaminants from a surface. Once the carbon bonds are broken down the reactive oxygen species created in the plasma can interact with the carbon and form additional bonds that can sterilise the surface (Donegan, Milosavljević and Dowling, 2013). Regarding the PDMS surface, this could explain the trend observed between the increase in oxygen and decrease in carbon (Figure 21) on the surface of the plasma treated PDMS and the close association between them.
This was also displayed graphically in Figures 22-24, which show representative high resolution analyses of silicon, carbon and oxygen atoms present on the surface of a representative PDMS 184 sample before and after plasma treatment. These Figures highlight the counts per second or the intensity of the peaks, the binding energies observed for each peak and also peak fitting, which also show the different binding energies of bonds that have formed following plasma treatment.

In Figure 22, the high resolution scan data for silicon was compared for untreated and oxygen plasma treated PDMS 184. The graph for untreated PDMS 184 in Figure 22 showed the silicon peak Si2p and also the two fitted silicon peaks (using Thermo Scientific Avantage™ data system) within it which were Si2p3 and Si2p1. The silicon (Si2p) binding energy shown for untreated PDMS 184 in Figure 22 was approximately 102 eV, which is commonly observed for organic PDMS (Thermo Fisher Scientific Inc, 2013). The two fitted peaks within the Si2p peak also had binding energies of 102 eV for Si2p3 and 102.88 eV for Si2p1, all binding energies confirming that it was untreated PDMS that was being measured. As the plasma treatment contained oxygen, it is known in the literature that the complex Si2p peaks of oxidised PDMS had been resolved into three components; Si bound to two oxygen atoms at 102.1 eV, Si bound to three oxygen atoms at 102.8 eV and Si bound to four oxygen atoms (SiO₂) at 103.4 eV (Hillborg and Gedde, 1999; Bodas, Rauch and Khan-Malek, 2008). Following plasma treatment, the high resolution surface analysis shown in Figure 22 was further de-convoluted to show the breakdown of the silicon peaks, due to the asymmetry of the Si2p peak as distinct separate peaks are not always observed. This showed the oxygen-silicon bond peak labelled as Si2p3 Ox at a binding energy of approximately 103.4 eV. The remaining peaks within Si2p were distinguished as Si2p3 for the PDMS (102 eV) and the Si2p1 peak was further separated into two peaks of approximately 103.9 eV and 102.6 eV, which was thought to be related to the silicon–oxygen bonds present after plasma treatment.

Figure 23 shows the high resolution scans for carbon on the surface of untreated and plasma treated PDMS 184. The untreated graph shows the peak for C1s which had a binding energy of 284.38 eV and the reference binding energy for C-C bonds is 284.8 eV (Moulder, 1992; Thermo Fisher Scientific Inc, 2013). In the plasma treated graph in Figure 23, the C1s peak was de-convoluted and fitted to distinguish the peaks observed after oxygen plasma on the surface of PDMS 184. A peak was identified at a binding energy of 284.28 eV which was thought to be produced by the C-Si bonds and at 285.1 eV, which was identified to be either C-H or C-C bonds (Moulder, 1992; Thermo Fisher Scientific Inc, 2013).

Figure 24 compared the high resolution data for oxygen on the surface of PDMS before and after plasma treatment. A key observation was the significant increase in the counts/s for the O1s peak
observed following oxygen plasma treatment, highlighting the increased deposition of oxygen reactive species onto the surface of the PDMS as functional groups incorporated into the PDMS polymer chains. For the untreated graph in Figure 24, the O1s peak had a binding energy of 532.2 eV and this was expected for oxygen present on the PDMS surface as SiO$_2$ is at 532.9 eV (Moulder, 1992; Thermo Fisher Scientific Inc, 2013) and oxygen from the atmosphere can also interact with the PDMS surface. Following plasma treatment, the O1s peak had a binding energy of 532.6 eV and was further separated and the O1s scan A was identified at 532.4 eV, which were indicative of SiO$_2$. Figures 21-24 determined that after oxygen plasma treatment, the surface chemistry of the PDMS had been successfully modified for further applications and the CAP plasma method had worked effectively.
Figure 19 – High resolution XPS analysis of Si2p silicon peaks on the surface of PDMS 184 comparing an untreated and oxygen plasma treated sample
Figure 20 - High resolution XPS analysis of C1s carbon peaks on the surface of PDMS 184 comparing an untreated and oxygen plasma treated sample
Figure 21 - High resolution XPS analysis of O1s oxygen peaks on the surface of PDMS 184 comparing an untreated and oxygen plasma treated sample.
In addition to XPS survey scans comparing the elemental composition of the PDMS surface before and after plasma treatment, XPS was also used to look at whole surface mapping of PDMS samples before and after plasma treatment. The distribution of the effects of CAP plasma treatment on the surface chemistry of the PDMS, specifically the distribution of oxygen atoms bound to the surface of the PDMS was observed.

Figure 25 shows the area map images for untreated and plasma treated PDMS and the change in the silicon, carbon and oxygen present on the surface of PDMS after plasma treatment. The shift between carbon and oxygen was more apparent, although following plasma treatment, it was observed that there was a heterogeneous distribution of oxygen atoms across the PDMS surface.

The right hand side of the sample shown in Figure 25 seemed to have lower oxygen binding compared to the rest of the surface. This could have been caused by the handling of the sample by tweezers and some exposure to the environment whilst setting up the XPS sample platter that holds that samples in place within the equipment. The distribution of the plasma treatment may have been affected by air flow within the cabinet that the plasma jet was in, which could have affected the direction of the plasma discharge from the jet and therefore the distribution of the oxygen atoms across the PDMS surface observed in Figure 25.

Overall the area maps showed that the plasma treatment had successfully modified the majority of the surface of the PDMS sample apart from some areas where the effects may have been reduced due to unavoidable handling of the PDMS between plasma treatment and XPS or air flow disruption to the plasma discharge. The XPS data obtained and shown in Figure 21 used survey scans which were carried out on three different areas on each PDMS sample tested. This enabled a more representative sampling method and between the different scans for each PDMS type, the standard deviation shown for each condition in Figure 21 was relatively low within the measurements for one type of PDMS. The area maps shown in Figure 25 gave an overall picture of the PDMS surface and showed a good distribution of bound oxygen following plasma treatment. It was ensured that plasma treated samples were handled as little as possible to avoid reducing the effects of plasma treatment. The hydrophobic recovery of plasma treated samples shown in Figure 20 has been reported in the literature after plasma treated PDMS has been exposed to air and it also is thought to be due to the diffusion of PDMS chains from the bulk of the material that have a low molecular weight. They move to the surface and replace the hydrophilic groups attached (Hillborg and Gedde, 1998; Berdichevsky et al., 2004). Therefore the plasma treated PDMS samples were carefully handled post treatment and stored in water for the rest of the sample processing steps as described in Section 3.2.1.1 of this Chapter.
Figure 22 – X-ray photoelectron spectroscopy area map carried out on untreated and plasma treated PDMS 184 showing the change in atomic percentage profile of Silicon, Carbon and Oxygen. Images are representative of the actual size of PDMS sample used and show the elemental composition over the whole surface of the sample being measured. XPS data analysis using the Thermo Scientific Avantage™ data system. Colour scales on all images ranging from 0 to 60% Atomic percentage.
3.2.2. Mechanical testing of PDMS

Following the preparation and surface modification of PDMS blends, the bulk mechanical properties were tested using three different methods; tensile testing, electronic speckle pattern interferometry (ESPI) and atomic force microscopy (AFM). Three methods were used in order to further understand the mechanical properties of each PDMS blend and how the variation between the different techniques also generated variation in the testing results for the Young’s modulus calculated from the data. PDMS has been used extensively for studies in mechanobiology and the effects of deformable substrates on cell behaviour (Brown, Ookawa and Wong, 2005; Fuard et al., 2008; Zhang et al., 2013; Carpi and Piel, 2014). In several applications, PDMS samples undergo a variety of loading conditions to test the bulk mechanical properties such as monotonic and cyclic loading, uniaxial and multiaxial testing and large deformations due to its elastic properties.

The bulk mechanical properties of the PDMS blends produced in this work was important in understanding how the changes in the substrate mechanical properties impacted on rabbit corneal epithelial (RCE) cell responses and behavioural changes and their mechanical properties, investigated further in the subsequent Chapters. The following Sections describe the different methods used on the PDMS blends and the data obtained from each technique, followed by a summary Section comparing the outcomes.

3.2.2.1. Tensile testing

Tensile testing was carried out using the Instron 3366 dual column tensometer or universal testing machine (Instron, UK) and the experimental parameters have been described in Chapter 2, Section 2.3.1. Figures 26-30 show the engineering stress-strain curves for PDMS 184, PDMS 10:1, PDMS 5:1, PDMS 1:1 and PDMS 1:5. The sample thickness ranged from 600-800 µm. The data shown has been limited to a strain of 0.3 for each PDMS blend, which was under 30% of the strain of the PDMS sample, in order to measure the linear part of the stress strain curve to calculate the Young’s modulus. As explained previously, at lower strain levels, it was assumed that the stress–strain behaviour was linear at small strains and therefore Young’s modulus could be calculated from the slope of the stress strain curve using Hooke’s Law, as described by equation 2 in Chapter 2, Section 2.3.1. The graphs show the average of three tensile tests per PDMS blend. Figures 26-30 show the area of each slope used to calculate the Young’s modulus and how the engineering stress and strain values were obtained. The graphs in Figures 26-30 show the average curve calculated from 3 samples in each PDMS condition.
Figure 23 – Engineering stress strain curve for PDMS 184 with linear fit and strain limited to 0.3, below 30% strain (mean ± SD, n=3).

Figure 24 – Engineering stress strain curve for PDMS 10:1 with linear fit and strain level limited to 0.3, below 30% strain (mean ± SD, n=3).
Figure 25 – Engineering stress strain curve for PDMS 5:1 with linear fit and strain level limited to 0.3, below 30% strain (mean ± SD, n=3).

Figure 26 – Engineering stress strain curve for PDMS 1:1 with linear fit and strain level limited to 0.3, below 50% strain (mean ± SD, n=3).
Figure 27 – Engineering stress strain curve for PDMS 1:5 with linear fit and strain level limited to 0.3, below 30% strain (mean ± SD, n=3).

From the engineering stress and strain values used to generate the average engineering stress strain curve for each PDMS blend in Figures 26-30, the Young’s modulus was calculated using a linear fit equation in Excel and the methods are described in more detail in Chapter 2, Section 2.3.1. Figure 31 shows the results obtained for Young’s modulus values from tensile testing of the PDMS blends.
It was found that there were no significant differences in the Young’s modulus values calculated between all PDMS blends from the tensile testing data obtained. The Young’s modulus values calculated from tensile testing data were; PDMS 184 = 0.28 ±0.06 MPa, PDMS 10:1 = 0.29 ± 0.04 MPa, PDMS 5:1 = 0.30 ±0.07 MPa, PDMS 1:1 = 0.22 ±0.03 MPa and PDMS 1:5 = 0.30 ±0.04 MPa. The trend observed in the data in Figure 31 did not follow the expected trend that the Young’s modulus would decrease with increasing amounts of Sylgard 527 (from pure PDMS Sylgard 184 to PDMS blend 1:5). However a decrease in Young’s modulus was observed after PDMS 5:1. There were no major differences between the PDMS blends but it was observed that the softest blend PDMS 1:5 had a high Young’s modulus, very similar to blends 10:1 and 5:1 (Figure 31). Both PDMS 184 and PDMS 1:5 did not follow the expected trend as PDMS 184 was thought to have exhibited the highest Young’s modulus and PDMS 1:5 the lowest Young’s modulus. This was due to the composition of the two samples as PDMS 1:5 had the least amount of Sylgard 184, which on its own forms a firmer gel when compared to pure Sylgard 527, which is too soft to use. Pure Sylgard 527 was not used as a PDMS sample due to its material properties and the difficulty in handling the polymer, making the process stages after PDMS preparation more challenging (see Chapter 2, Section 2.1.2).

It was observed in the stress strain measurements obtained from tensile testing that PDMS 1:1 strain response or deformation (Figure 29) was different to the other PDMS samples tested as well as the
difference in Young’s modulus observed (Figure 31). In Figures 26-30 the strain data plotted in the graphs was limited to a strain of 0.3, which for all PDMS blends except for PDMS 1:1 was below 30\% strain. However, PDMS 1:1 responded differently to the load exerted and this meant that at 0.3 strain the data was already just below 50\% strain. This showed that the changes in the polymer composition generated distinct differences in the mechanical responses of the PDMS samples to load and their strain rates. As previously described in Chapter 2, Section 2.1.2, there are some differences in the compositions of the two types of Sylgard used to produce the PDMS blends. Sylgard 184 and 527 are made from the same material primarily however Sylgard 184 contains additional chemicals and silica nanoparticles, which can significantly increase the stiffness of the polymer (Oláh, Hillborg and Vancso, 2005; Dow Corning, 2016a, 2016b, 2016c, 2016d). Therefore the mechanical properties of the PDMS blends would have been affected dependent on the different ratios of Sylgard 184 to Sylgard 527.

Several factors were thought to have contributed to the results obtained in Figure 31. Firstly the PDMS blends were prepared by weight and were mixed by hand which would have increased the variability of the PDMS preparation process. Therefore the thicknesses of the PDMS substrates were not uniform and variability in the thickness could affect the bulk mechanical property measurements obtained. However, the thickness of the PDMS blends had previously been measured and they ranged between 600–800 µm and the weight of each component of the polymers when being mixed was kept the same each time. A thickness below 200 µm was found to be the point at which PDMS transitioned between bulk mechanical properties and thickness dependent mechanical properties (Liu et al., 2009). Liu et al (2009) also carried out tensile testing on PDMS with different thicknesses using the ASTM standard test for vulcanised rubber and thermoplastic elastomers and they found that below 200 µm there is a critical change in the PDMS that triggers the reordering of the polymer chains. It was thought that this structural change that was induced by the thickness of the PDMS is related to the shear stress produced in the sample during fabrication, and the reorganisation of polymer chains that increased the mechanical strength of the PDMS and cross-linked networks (Liu et al., 2009).

Different definitions of stress strain curves are found in the literature when determining the elasticity of a material or human tissue, which are engineering stress strain curve or true stress engineering strain curve. However these curves are not indicative of the deformation characteristics of a PDMS elastomer because they are based on the original dimensions of the elastomer and the dimensions change continuously during the test (Khanafer et al., 2009). By using true stress and true strain curves, a more accurate result of the stress strain curve for the tensile data could be achieved.
It was found in PDMS 184 that the engineering stress strain curve and the true stress engineering curve were very similar to the true stress strain curve at small strains. However at larger strains, the true stress strain curve can be much greater that the engineering stress strain curves. Also the Young’s modulus for true stress strain curves is significantly higher at higher strains when compared to engineering curves (Fuard et al., 2008; Khanafer et al., 2009; Liu, Sun and Chen, 2009). Therefore as engineering stress strain curves were used to analysis the Young’s modulus of the PDMS samples (Figures 26-31); the values obtained were likely to be lower than the true stress and strain and therefore the Young’s modulus. Further analysis would be required to determine true stress and strain but the data on engineering stress and strain has enabled a further understanding of the bulk mechanical properties of the PDMS blends.

The Young’s modulus value of PDMS is highly dependent on the definition of stress and strain and whether engineering or true stress strain curves are used. It is also dependent on the strain rate and the mixing ratio of PDMS. However in the work carried out in this thesis, PDMS 184 mixing ratio of 10:1 (elastomer base: curing agent) was not changed as this was the optimum mixing ratio to ensure no leaching of free polymer that had not been cross-linked during the curing process (Palchesko et al., 2012). The "engineering" stress strain curves shown in Figures 26-30 used tensile strain (mm/mm) calculated by the Instron 3366 software as the extension (mm) divided by the gage length of the PDMS sample of 30 mm, which was the original gage length at the starting point of the tensile test. The linear regression of the slope of the stress strain curves was used for each PDMS blend and the data used was limited to 0.3 strain. The methods used may not have been a true representation of the stress strain curves for PDMS as it is not a true reflection of the change in dimensions of the test piece under tensile load. As explained previously, there were differences in the strain rates, which were more pronounced in the stress strain data for PDMS 1:1 compared to the other PDMS blends tested. Tensile testing may not have been sensitive enough to detect changes between the PDMS blends at small strains. Although tensile testing is still a widely used test for mechanical properties of PDMS, it does not take into account the dynamic deformation and mechanical properties of the PDMS and elastic or viscoelastic shear characteristic (Carey et al., 2011; Bhamra and Tighe, 2017).

Following the tensile testing experiments, PDMS 1:5 was no longer used in further mechanical tests going forward. This was due to the following techniques and the difficulty in handling this PDMS blend. Also in Chapter 4, PDMS 1:5 was shown to be an unfavourable surface for RCE cell attachment and cell migration and later Chapters focus on a smaller range of PDMS blends.
3.2.2.2. Electronic speckle pattern interferometry (ESPI)

Following tensile testing of PDMS blends, a more accurate, highly sensitive measurement to calculate the Young’s modulus of the PDMS blends was carried out using electronic speckle pattern interferometry (ESPI). To obtain these results, experiments were carried out using a rig designed by Abby Wilson (Wilson, 2017). The experiments were also conducted by and data processed by Abby Wilson to obtain the maximum deflection values used to calculate Young’s modulus. The equations and methods used to calculate Young’s moduli are explained in more detail in Chapter 2, Section 2.3.2. Table 4 shows the maximum deflection data obtained from ESPI and the converted values for Young’s modulus calculations.

Table 4 – Maximum deflection values for each PDMS blend measured using ESPI (courtesy of Abby Wilson). Values converted to metres after data was obtained.

<table>
<thead>
<tr>
<th>PDMS</th>
<th>Maximum Deflection (Y_m) (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>184</td>
<td>1.0233 x 10^{-6}</td>
</tr>
<tr>
<td>10:1</td>
<td>1.0337 x 10^{-6}</td>
</tr>
<tr>
<td>5:1</td>
<td>1.1652 x 10^{-6}</td>
</tr>
<tr>
<td>1:1</td>
<td>0.6509 x 10^{-6}</td>
</tr>
</tbody>
</table>

Figure 32 shows the Young’s modulus values obtained from the ESPI experiments. Calculations were carried out using the provided maximum deflection data to work out the Young’s modulus value and the four PDMS blends that were measured were PDMS 184, 10:1, 5:1 and 1:1. This was due to the difficulty of handling the softest PDMS blend 1:5 and the setup within the rig used for ESPI experiments. Due to the constraints of the material, data could not be obtained for this blend.
The results obtained from ESPI showed that the Young’s modulus values calculated from the maximum deflection were; PDMS 184 = 3.85 MPa, PDMS 10:1 = 5.21 MPa, PDMS 5:1 = 6.27 MPa and PDMS 1:1 = 7.29 MPa. These values were found to be very different from the tensile testing and AFM data (Sections 3.2.2.1 and 3.2.2.3 respectively). However, the Young’s moduli obtained for the PDMS blends tested using ESPI did not follow the expected trend, which was with increasing amounts of Sylgard 527, the Young’s modulus would decrease as described previously. The mechanical properties were expected to change and decrease with increasing amounts of Sylgard 527 due to changes in the chemical composition and the reduction of the silica nanoparticle fillers that are present in Sylgard 184 and not in Sylgard 527 (Palchesko et al., 2012). Different measurements were used with this technique and the data collected by Abby Wilson was used to calculate the maximum deflection of the PDMS sample being tested. This was then used in the data shown in Figure 32 to obtain the Young’s modulus. However, for the ESPI data, measurements were not repeated and therefore have to be further investigated and more tests need to be carried out in order to calculate the Young’s modulus of several samples. The trend observed in Figure 32 may have been influenced by the thickness of each PDMS sample used to calculate the data presented in Figure 32. The PDMS samples used in this experiment ranged from 600–800 µm and was different for each PDMS blend. Further work is needed to standardise the process in terms of PDMS sample
thickness as this can affect the resulting Young’s modulus calculation, to optimise the ESPI protocol and to reproduce the data. ESPI is a very sensitive method, non-contact, full-field measurement system and can be used to measure the mechanical properties of biological tissues such as the cornea (Zhang et al., 1998; Yang et al., 2014; Wilson, Marshall and Tyrer, 2016). Further optimisation of this method for PDMS samples is required for future work and development of their own standardised protocols for the testing of PDMS substrates.

3.2.2.3. Atomic force microscopy

Atomic force microscopy was also used to measure the mechanical properties of the PDMS blends. The apparent Young’s modulus of each PDMS sample without RCE cells cultured on the surface was calculated following QI™ imaging. For the AFM work carried out four PDMS sample types were chosen; PDMS 184, PDMS 10:1, PDMS 5:1 and PDMS 1:1. This was due to the limitations of the softest blend PDMS blend 1:5, as described earlier in Chapter 2, Section 2.1.2. The methods used for AFM and data analysis are detailed in Chapter 2, Section 2.4. Figure 33 shows the average Young’s modulus for each PDMS type as measured using the AFM.

![Figure 30 - Average Young’s modulus of different PDMS types using QI™ data and Hertz fit (JPK Data Processing, JPK Instruments) (mean ± SD, n=3). ** and *** show a significant decrease in the Young’s modulus in PDMS 10:1 (p ≤ 0.01), PDMS 5:1 (p ≤ 0.001) and PDMS 1:1 (p ≤ 0.001) when compared to PDMS 184. Statistical analysis carried out using One-way ANOVA, Tukey analysis, Minitab.](image-url)
The results in Figure 33 showed that there were significant differences between the average Young’s modulus of PDMS 184 when compared to PDMS 10:1, PDMS 5:1 and PDMS 1:1 (ratio of Sylgard 184 to Sylgard 527) and PDMS 184 had a significantly higher Young’s modulus compared to the rest of the PDMS blends tested. Therefore, as the mass ratio of Sylgard 527 increased in the PDMS blends, the Young’s modulus of the substrate decreased. The data showed that the average Young’s modulus for the samples were; PDMS 184 = 59.45 ±8.84 MPa, PDMS 10:1 = 22.92 ±17.13 MPa, PDMS 5:1 = 7.79 ±6.88 MPa and PDMS 1:1 = 4.27 ±1.60 MPa.

It has been previously shown that combining these two types of Sylgard can produce a range of PDMS substrates with different Young’s moduli (Palchesko et al., 2012). The values obtained for Young’s modulus using AFM followed the expected trend however the average Young’s modulus obtained for PDMS 184 was much greater than those values reported in the literature of around 1–3 MPa (Mata, Fleischman and Roy, 2005; Wang et al., 2010; Johnston et al., 2014). This discrepancy could have been due to the different methods that are used to obtain the Young’s modulus data and in this case, the data has been obtained using nanoscale technology that measures the surface interactions rather than the bulk mechanical properties.

A change from macroscale to microscale testing was expected to show differences in Young’s modulus due to the nature of the tests used (Garcia-Porta et al., 2014; Sharfeddin et al., 2015). This allowed the mechanical properties to be analysed at the scale relevant to the size of the cells, relating to the changes in the substrates that the cell would detect through mechanosensing (Wen et al., 2014). However, it has been reported that from macroscale to microscale testing methods, values for Young’s modulus reduced (Sharfeddin et al., 2015). In the results shown in Figure 31 and Figure 33, tensile testing produced a lower value for Young’s modulus of the PDMS blends (Figure 31) when compared to the AFM results for Young’s modulus, which were significantly higher (Figure 33). This could have been due to the use of different techniques and equipment used to obtain the data and also in terms of the analysis of this data. Tensile testing data used engineering stress and strain to calculate the Young’s modulus whereas the JPK NanoWizard® Data Processing (DP) software (Chapter 2, Section 2.4.5 and 2.4.6) converted the force curves to correct for cantilever bending and force against tip-sample separation was plotted and the Hertz model was fitted to each curve using the software and the resulting Young’s modulus data was obtained. The data processing software allowed the quantitative measurement of the nanoscale material stiffness and all the force-distance curves obtained could be used in data processing following imaging.

Also the methods used to produce the PDMS blends throughout the experiments were done using different methods of curing and preparation as described previously in the materials and methods.
Chapter (Chapter 2, Section 2.1) and there is no single standard method of preparing PDMS, as shown in the literature (Palchesko et al., 2012; Johnston et al., 2014). Therefore a direct comparison of the Young’s modulus data would not be appropriate as heat curing for example can significantly affect the mechanical properties of the cured PDMS along with other process parameters such as mixing ratios and strain rates (Khanafer et al., 2009; Liu, Sun and Chen, 2009; Johnston et al., 2014).

Although the apparent or estimated Young’s modulus was obtained from the analysis of the AFM data, the PDMS samples fitted the assumptions of the Hertz model as they are an isotropic solid. Also Poisson’s ratio was applied in the model and it is normally approximately 0.5 for PDMS (Teixeira et al., 2009). The use of this model has been justified previously in Chapter 2, Section 2.4.6 and although there are assumptions to the model, it is commonly used to calculate the Young’s modulus of soft biological samples and substrates (Verdier, 2003; Valero et al., 2016).

The decrease in Young’s modulus with decreasing amounts of PDMS 527 was expected as mentioned previously, Sylgard 184 contains silica nanoparticles as fillers that reinforce the cured polymer hence producing a stiffer material with a higher Young’s modulus (Dow Corning, 2016a, 2016b, 2016c, 2016d). In the curing process, the vinyl of the silica filler and the silicon hydride groups from the curing agent go through a hydro-silylation reaction to form Si-C bonds and cross linking occurs (Lee et al., 2004; Liu et al., 2015). As the amount of Sylgard 527 increased, the PDMS blends were therefore expected to decrease in stiffness or Young’s modulus as Sylgard 527 does not contain any silica fillers or other reinforcing components. The chemical reaction of cross linking would have less of an effect on the overall bulk mechanical properties of the PDMS blend as the amount of PDMS 184 decreased (Palchesko et al., 2012; Dow Corning, 2016c, 2016d).

Another property of PDMS that was not investigated was the swelling of the PDMS substrates as they were stored in distilled water and were used to culture RCE cells and were kept in cell culture medium. This is a limitation of PDMS but it is the use of organic solvents that can cause swelling of the PDMS and a decrease in the stiffness as the organic solvent is taken up by PDMS, changing the structural properties of the polymer (Zhou et al., 2012; Johnston et al., 2014). By using aqueous solutions, swelling of the PDMS can be avoided (Zhou et al., 2012) however following plasma treatment the surface of the PDMS was hydrophilic. Further investigations would be needed to investigate if swelling did occur due to this or if the duration the PDMS samples were in distilled water did not cause significant swelling that could affect the bulk mechanical properties of the PDMS samples.
AFM has been used more recently to test the mechanical properties of soft biological materials even though more traditional macroscopic methods such as uniaxial tensile testing of materials are commonly used (Valero et al., 2016). However extremely soft biological materials or for example the soft PDMS blend described previously cannot be tested properly using tensile tests and techniques such as AFM and other non-destructive methods have been employed to determine the mechanical properties of soft materials.

AFM performs nanoindentation tests on the material and can measure nanoscale forces in real time (methods used are described in more detail in Chapter 2, Section 2.4). It is a technique that has been used to characterise soft biological materials with a low Young’s moduli that can be challenging to ascertain the mechanical properties of due to biological tissue being non-homogenous and anisotropic. This is because the Hertz model assumes the material is isotropic and homogenous but it is still one of the most commonly used methods to estimate the Young’s modulus from AFM force–displacement data and this method is applied depending on the material properties and indenter geometry. AFM and nanoindentation can measure small sample sizes and can apply very small deformations to the material, allowing for small structures to be characterised. Nevertheless, this means that AFM is required to operate at high precision due to the sensitivity of the technique (Valero et al., 2016). In terms of the data shown in Figure 33, the high Young’s modulus values calculated could be related to the high sensitivity of the AFM instrument and also the mode used for measurements was QI™ mode, an imaging mode that does not apply any lateral forces and allows a precisely controlled vertical force at each pixel whilst imaging (JPK Instruments AG, 2011). There were several factors to consider with the AFM data as well as the other methods of mechanical testing of the PDMS blends. Although the resulting Young’s moduli found for each blend were not directly comparable to one another, they provided further information on the bulk and surface mechanical properties of the PDMS blends that were generated using two types of Sylgard and different preparation methods compared to the literature, as described in Chapter 2, Section 2.1 (Mata, Fleischman and Roy, 2005; Liu et al., 2009; Palchesko et al., 2012; Deguchi et al., 2015; Placet and Delobelle, 2015). It is clear that the process used to produce PDMS substrates of different Young’s moduli can differ greatly and this can have a major impact on the bulk mechanical properties of the substrate. The testing methods and data analysis methods in this Chapter have shown large differences in the Young’s moduli obtained ranging over orders of magnitude. This highlights the impact of different processes on the final PDMS substrate mechanical properties.
3.3. General discussion and conclusions

Overall, the PDMS blends underwent surface modification in the form of CAP plasma treatment to produce a more hydrophilic surface. This was tested for its effectiveness and also in terms of the durability of the treatment using water contact angle measurements and XPS. It was found that the more hydrophilic surface was successfully produced on the PDMS samples using oxygen CAP plasma and by storing the plasma treated sample in distilled water immediately after treatment, the rate of recovery of the hydrophobic nature of the PDMS surface was slowed as compared to plasma treated PDMS samples that were kept in air after treatment.

The PDMS blends were then tested for their mechanical properties using three different methods, tensile testing, ESPI and AFM. The Young’s modulus values obtained from the different testing methods varied greatly and over orders of magnitude. For example, for PDMS 184 the value of Young’s modulus calculated from tensile testing data was PDMS 184 = 0.28 ±0.06 MPa. For ESPI data the Young’s modulus calculated from the maximum deflection for PDMS 184 was 3.85 MPa, and for AFM, the Young’s modulus was 59.45 ±8.84 MPa. It was thought that this was due to the different testing methods used and the different methods for analysis of Young’s modulus required for each test and differences in macroscale and microscale testing methods have been discussed previously and in the literature (Garcia-Porta et al., 2014; Sharfeddin et al., 2015).

In terms of the corneal biomechanical properties, the Young’s moduli, measured in vitro can range from 0.1–57 MPa in the literature (Elsheikh, Alhasso and Rama, 2008b; Garcia-Porta et al., 2014). The PDMS samples produced may have fallen within a large range of Young’s moduli values that have been observed in corneal tissue, reported in the literature. This was a physiologically relevant range but there was drastically high variation in Young’s modulus calculated from tensile tests, ESPI and AFM. However the variation between PDMS samples tested for the same PDMS blend in the tensile tests was low compared to AFM.

The differences in the Young’s modulus values obtained in this research when compared to similar materials in the literature was caused by different preparation methods and processes used to produce PDMS substrates and blending two different types of Sylgard at differing ratios. For microfabrication techniques, spin coating of the PDMS is a commonly used procedure to form films of a uniform thickness (Mata, Fleischman and Roy, 2005). Also for mixing of the PDMS polymer before curing, commercial mixers have been used and the viscous mix is then degassed in a vacuum desiccator to ensure that the mix is bubble free before curing. This is more important if using heat curing as the bubbles become trapped with the heat and form an non-homogenous PDMS film (Johnston et al., 2014). These factors affect the cured PDMS bulk mechanical properties and the
processes used to prepare the PDMS samples in this work (see Chapter 2, Section 2.1) were very different to some of the methods used in literature, as there is not one standard method to prepare PDMS. This can also be an advantage because the PDMS substrates can be fine-tuned to physiologically relevant Young’s moduli similar to biological tissues.

Sensitive testing methods are needed for softer materials and biological tissues but they need to be very precise in their measurements the higher the sensitivity. Macroscale testing methods may not be able to measure very low stiffness of a material or biological tissue and the practicality of using these methods with very soft material would not be feasible.

In conclusion, the production of PDMS substrates with different Young’s moduli was achieved and the PDMS substrate surfaces were successfully modified to increase their hydrophilicity and generate a more attractive surface for RCE cell attachment. The range of Young’s moduli produced from the AFM data showed the trend of Young’s modulus that was expected in that an increasing ratio of Sylgard 527 produced a less stiff PDMS substrate and decreased the Young’s modulus of the substrate. The Young’s modulus results showed the variability across different testing methods and analysis methods used and with the more sensitive techniques, ESPI and AFM, the expected trend was only observed in the AFM results and not in the tensile test used. The ESPI results showed the opposite trend to what was expected but the variations in the thickness of each PDMS blend measured would have impacted on the final calculations. Therefore further work is required to establish protocols for this technique and standardisation of the methodology used to generate PDMS samples in order to obtain the same thickness each time is needed. This highlights that the sensitivity of the method used could provide different mechanical properties and can differentiate between more subtle changes in material stiffness. In the following Chapter, RCE cell attachment, morphology and cell migration were investigated to correlate different PDMS substrate stiffness to changes in cellular responses.
Chapter 4

Rabbit Corneal Epithelial Cell Culture on Polydimethylsiloxane Substrates
4.1. Introduction

Cells are known to respond to mechanical cues or stimulation in their surrounding extracellular environment and these mechanical changes have a powerful influence on cell behaviour (Angelini et al., 2010). Types of cell behaviour that are regulated by mechanical cues in the micro-environment of the cell are cell spreading, migration, proliferation and differentiation (Huang et al., 2012). The cytoskeleton of the cell plays a key role in the mechanics of a cell and how the cytoskeleton and extracellular matrix interactions that are related to the function, shape, deformability and mechanical properties of a cell are an important factor in studies of cell mechanics (Zhu, Bao and Wang, 2000).

The mechanical stimulation of mammalian cells has been shown to affect cell functions such as cell motility, proliferation and apoptosis. The cells response to mechanical stimulation also causes changes in protein and gene expression and this biochemical response to physical excitation of cells is known as mechanotransduction (Steward et al., 2010). Matrix stiffness has been shown to have a strong influence on cell adhesion and organisation and there are key cellular processes that have been shown to be dependent on the adhesion geometry and matrix stiffness (Schwarz and Gardel, 2012), which can affect cell survival and stem cell differentiation (Engler et al., 2006; Kilian et al., 2010). Well-defined mechanical and surface properties of substrates used for tissue constructs can have an impact on the success of a tissue construct in terms of the cell number and differentiation of cells (Wong, Leach and Brown, 2004; Jones, Hamley and Connon, 2012).

Spreading and cell migration can also be influenced by the mechanical properties or stiffness of a substrate (Petroll and Miron-Mendoza, 2015). Cell migration has a critical role in many physiological processes such as the immune response, morphogenesis, apoptosis and wound healing (Sears and Kaunas, 2016). Cell migration can also be guided by a stiffness gradient known as durotaxis and it has been demonstrated that physical cues alone can direct cell movement (Lo et al., 2000).

In the previous Chapter, PDMS blends had been prepared that had a range of different Young’s moduli and their bulk mechanical properties were tested using three different methods. Surface characterisation of the PDMS blends was also carried out following successful surface modification to produce a more attractive surface for cell adhesion. In this Chapter, the effects of the PDMS blends with differing Young’s moduli were tested and used as substrates for the culture of Rabbit corneal epithelial (RCE) cells. The effect of different substrate stiffness was tested on RCE cell adhesion, spreading, RCE cell viability and cell migration to further investigate the physical responses of the RCE cells to mechanical changes in their surrounding environment.
4.1.1. Aims and Objectives

The aim of this Chapter is to further investigate the PDMS samples prepared for cell culture and to see how the differing bulk mechanical and surface properties of the PDMS blends affect cell attachment, spreading, proliferation and migration. The objectives to achieve the aim are to culture RCE cells on the surface of untreated and plasma treated PDMS blends and quantify cell attachment using an automated imaging platform, the Nikon BioStation CT. A further objective is to measure RCE cell viability and proliferation during culture on PDMS substrates in comparison to tissue culture plastic and to investigate how the difference in substrate mechanical properties affect cellular processes and behaviours. Cell migration will be measured and quantified also using the BioStation CT and will be compared across the different PDMS blends to see how changes in substrate properties affect cell migration.

4.1.1.1. Hypothesis

It is hypothesised that following plasma treatment of the PDMS substrate surface, RCE cell attachment will be higher than on untreated PDMS surfaces. Following culture of RCE cells on the surface of the PDMS blends, the differing mechanical properties of the PDMS substrates will have an effect on cell behaviours. In terms of RCE cell attachment, viability, proliferation, and migration, it is hypothesised that as the ratio of Sylgard® 527 compared to Sylgard® 184 increases, there will be a decrease in these cell behaviours due to the decreasing stiffness of the PDMS blends.
4.2. Results

An RCE cell line was used in experiments and cultured following the methods described in Chapter 2, Section 2.5. The RCE cell line used was an immortalised cell line in which the primary RCE cells had been genetically modified with a recombinant viral vector SV40. This allowed continuous culture of the RCE cells and a continued proliferation and growth over several generations and passages without phenotypic changes (Araki et al., 1993). The RCE cell line provided a research tool that was used to establish protocols with an easy to handle cell line in vitro without the setbacks of primary corneal cell culture such as a lower cell yield, heterogeneity in culture, and a poor proliferative ability and therefore short life span (Araki et al., 1993). The justification of using this cell line was that the RCE cell line was chosen for process development. As they were not primary cells such as limbal stem cells, this provided an easier way to develop methods and for the optimisation of the processes used. This allowed proof of concept experimental work and further development of techniques used that would be applied to other cell lines or primary cells in future work.

4.2.1. RCE cell culture on PDMS

The appearance of the RCE cells when cultured on untreated PDMS, plasma treated PDMS and on tissue culture plastic (TCP) under normal conditions of 37 °C, 5 % CO₂ in RCE cell culture medium can be seen in Figure 34. This Figure shows the effects of the CAP oxygen plasma used on PDMS samples (discussed in Chapter 3, Section 3.2.1). Images of the RCE cells were taken over three days in culture and the RCE cells cultured on untreated PDMS formed attachment on day 1 and day 2 of culture. However by day 3 it was apparent that the RCE cells in this condition were detaching from the surface of the PDMS and forming clumps of cells on the surface or floating in the culture medium. This showed that the PDMS without plasma treatment had an unfavourable surface for RCE cell attachment.

In order to produce a more hydrophilic and therefore a more attractive surface for RCE cell attachment, CAP plasma was used and it had been shown to successfully modify the surface to become more hydrophilic and suitable for cell culture. This can be observed in the plasma treated PDMS images in Figure 34. The plasma treated sample cultured over 3 days with RCE cells showed the success of the CAP oxygen plasma used for PDMS surface modification (discussed in Chapter 3, Section 3.2.1). The morphology of the RCE cells appeared to be very similar to RCE cells grown on TCP and also the attachment and proliferation of the RCE cells was observed to increase over the 3 day culture. This showed that the CAP plasma treatment had worked effectively, supporting the data on surface modification in Chapter 3.
The typical cobblestone-like morphology of this cell line was observed especially on days 2 and 3 in culture when RCE cells were cultured on plasma treated PDMS and TCP. Following this, the PDMS surface and bulk mechanical properties were tested to see how they would affect RCE cells in terms of their attachment, morphology and spreading on the surface, proliferation and growth and the effect of the different PDMS substrate properties on RCE cell migration.

Figure 31 - RCE cells cultured on PDMS and on tissue culture plastic over 3 days in culture. The change in cell morphology and attachment between untreated and plasma treated PDMS compared to tissue culture plastic was observed. Images taken at 10x magnification using the Nikon inverted microscope. Scale bar = 100µm.
4.2.1.1. RCE cell attachment on PDMS blends and staining for F-actin

Further investigation into the attachment of RCE cells on the different PDMS blends was carried out. In order to do this, RCE cell–PDMS samples were fixed after 24 hours in culture and fluorescently stained for a key component of the cytoskeleton, the filamentous actin (F-actin). These methods are described in more detail in Chapter 2, Section 2.6.

Actin is one of the major components present in the cytoplasm of eukaryotic cells and it occurs in two forms; a globular form or G-actin and F-actin, which is the filamentous form. The F-actin forms polarised filamentous bundles that are known as stress fibres. F-actin also forms microfilaments which can be found as a fine network underneath the cell plasma membrane (Ndozangue-Touriguine, Hamelin and Bréard, 2008; Papakonstanti and Stournaras, 2008). The stress fibres or F-actin bundles are critical for maintaining the connection between the cell and the surface on which it is growing and were therefore deemed important in these attachment studies.

The PDMS blends that were studied were PDMS 184, PDMS 10:1, PDMS 5:1, PDMS 1:1 and PDMS 1:5 (ratio of Sylgard® 184: Sylgard® 527). Images were taken of the PDMS-RCE cell samples that had been stained on the Nikon Ti Eclipse inverted fluorescent microscope. Figures 35-39 showed two representative images of RCE cells cultured on a particular PDMS sample, showing untreated and plasma treated conditions in brightfield and fluorescent images. The images selected for plasma treated samples showed an area where fewer RCE cells were attached as well as an area where a higher number of RCE cells were attached, showing individual cells and clusters of cells.
Figure 32 – RCE cells cultured on PDMS 184 and stained with Phalloidin for F-actin in the cytoskeleton and DAPI nuclear stain at 20x magnification. Representative brightfield and fluorescent images taken of RCE cells cultured on untreated PDMS 184 (A-D, 2 samples) and on plasma treated PDMS 184 (E-H, 2 samples). Scale bar = 100µm. Images taken using the Nikon Eclipse Ti fluorescence microscope and digital camera.
Figure 33 – RCE cells cultured on PDMS blend 10:1 and stained with Phalloidin for F-actin in the cytoskeleton and DAPI nuclear stain at 20x magnification. Representative brightfield and fluorescent images taken of RCE cells cultured on untreated PDMS 10:1 (A-D, 2 samples) and on plasma treated PDMS 10:1 (E-H, 2 samples). Scale bar = 100µm. Images taken using the Nikon Eclipse Ti fluorescence microscope and digital camera.
Figure 34 – RCE cells cultured on PDMS blend 5:1 and stained with Phalloidin for F-actin in the cytoskeleton and DAPI nuclear stain at 20x magnification. Representative brightfield and fluorescent images taken of RCE cells cultured on untreated PDMS 5:1 (A-D, 2 samples) and on plasma treated PDMS 5:1 (E-H, 2 samples). Scale bar = 100µm. Images taken using the Nikon Eclipse Ti fluorescence microscope and digital camera.
Figure 35 – RCE cells cultured on PDMS blend 1:1 and stained with Phalloidin for F-actin in the cytoskeleton and DAPI nuclear stain at 20x magnification. Representative brightfield and fluorescent images taken of RCE cells cultured on untreated PDMS 1:1 (A-D, 2 samples) and on plasma treated PDMS 1:1 (E-H, 2 samples). Scale bar = 100µm. Images taken using the Nikon Eclipse Ti fluorescence microscope and digital camera.
Figure 36 – RCE cells cultured on PDMS blend 1:5 and stained with Phalloidin for F-actin in the cytoskeleton and DAPI nuclear stain at 20x magnification. Representative brightfield and fluorescent images taken of RCE cells cultured on untreated PDMS 1:5 (A-D, 2 samples) and on plasma treated PDMS 1:5 (E-H, 2 samples). Scale bar = 100µm. Images taken using the Nikon Eclipse Ti fluorescence microscope and digital camera.
Figures 35-39 show the morphology of the RCE cells when cultured on the surface of the different PDMS blends. Each Figure shows representative images of the RCE cells cultured on the different PDMS blends, with one image showing a less populated area and the other image showing an area with a higher number of RCE cells in the plasma treated condition. Changes in morphology were apparent when the images from PDMS 184 to PDMS 1:5 were observed, with notable differences in morphology of RCE cells cultured on PDMS blend 1:5, which had the lowest amount of PDMS 184 and contained more of the softer PDMS, Sylgard 527. The mechanical properties of the PDMS blends measured in Chapter 3 showed that with increasing amounts of Sylgard 527, the Young’s modulus of the PDMS blend decreased and the PDMS substrate decreased in stiffness. This correlated to the results observed in Figures 35-39 and showed that the mechanical properties of the PDMS blends were clearly impacting on the cell attachment and morphology of the RCE cells on the PDMS substrates.

The change in morphology observed in the RCE cells was that as the Young’s modulus of the PDMS blend decreased, the shape of the cytoskeleton changed and became less spread out and more rounded. This indicated that the RCE cells were unable to spread across the surface of the PDMS and form focal adhesions. Cell adhesion is highly dependent on substrate stiffness and it has been shown that cell types that are cultured on very soft substrates cannot form stress fibres or elongated adhesions, and these are important for maintaining the cell-matrix attachments. On stiffer glass or plastic substrates, cells are more likely to produce larger focal adhesions and stress fibres (Schwarz and Gardel, 2012). The change in shape was observed in the softer PDMS blends as the RCE cells appeared more rounded and did not form elongated structures. This was much more apparent in PDMS 1:5 (Figure 39) and was not as obvious in PDMS 1:1 (Figure 38) however, the area imaged where there were not many RCE cells on PDMS 1:1 seemed to have RCE cells with more rounded cell bodies, indicating cell detachment and lack stress fibre formation. On PDMS 184, PDMS 10:1 and PDMS 5:1 (Figures 35-37) RCE cells were observed to have a similar morphology and the cell bodies of the RCE cells appeared larger and more spread compared to PDMS 1:5 (Figure 39), with actin fibres in the cytoskeleton clearly visible in the images.

It was found that the softest PDMS blend with the lower Young’s modulus had a much lower number of RCE attached, which was later quantified using the BioStation CT. This could have been caused by the bulk mechanical properties affecting the attachment of RCE cells to the surface of the PDMS. The topography, stiffness, chemical functionality and the adhesive ability of the cells to the surface are some of the properties of the cell-material interface that can affect how cells bind to the extracellular matrix (ECM) through integrins and other cell adhesion molecules (Murphy, McDevitt
Another factor that was considered with the PDMS blends and cell attachment was that with increasing amounts of Sylgard 527, the PDMS blends contained less of the Sylgard 184, which contained fumed silica nanoparticles that reinforced the polymer structure when cured hence the stiffer PDMS (Lee et al., 2004; Palchesko et al., 2012). Sylgard 527 does not contain silica nanoparticles (Dow Corning, 2016c, 2016d) and therefore forms PDMS with a lower Young’s modulus and is less stiff as the ratio of Sylgard 527 increases. This affected the Young’s modulus of the PDMS blends prepared as shown in Chapter 3 and the effects of this can be observed through the F-actin fluorescent staining in Figures 35-39.

PDMS 1:5 was difficult to handle and manipulate within the mechanical tests carried out on the PDMS blends as discussed in Chapter 2, Section 2.1.2 and Chapter 3, Section 3.2.2.1, and it was not used in two of the mechanical tests, ESPI and AFM. However actin staining of RCE cells cultured on PDMS 1:5 was carried out as was XPS following CAP oxygen plasma treatment (Chapter 3). The XPS results shown in Chapter 3 (Figure 21) showed that after plasma treatment, PDMS 1:5 had a much lower atomic percentage of oxygen at 38% whereas the other PDMS blends tested all had an atomic percentage of oxygen greater than 45%. It was also observed and discussed in Chapter 3 that the carbon and oxygen were linked and the increase in oxygen following plasma treatment correlated with a decrease in carbon. PDMS 1:5 also had a higher atomic percentage of carbon present on the surface compared the other PDMS blends, which had all decreased with the increase in carbon following plasma treatment. This data along with the F-actin staining images of RCE cells cultured on PDMS 1:5 (Figure 39) suggested that this particular PDMS blend was not an optimum composition to produce a substrate suitable for RCE cell culture and did not have the mechanical and surface properties to attract RCE cell attachment and support the proliferation and spreading of the cells. This may have also been due to the increased amount of Sylgard 527 forming a layer on the surface of this blend of PDMS oligomers that have not cross-linked and can move to the surface of the PDMS (Kim et al., 2010; Wang et al., 2010). An oil-like layer can form, which may have reduced the effects of the plasma treatment, as shown by the XPS data (Chapter 3, Figure 21). Therefore, this would have maintained more of a hydrophobic surface, causing RCE cells to detach or less attachment in the first instance.

These differences in the morphology of the cytoskeleton of the RCE cells, fluorescently stained using Phalloidin, suggested that on the softer two blends of PDMS, PDMS 1:5 and 1:10, RCE cells were still able to form a weak attachment to the surface of the PDMS following plasma treatment. However, the morphological changes also suggested that the RCE cells were unable to develop anchorage points to the surface of the PDMS via actin stress fibres and adhesion molecules such as integrins.
Anchorage through these transmembrane receptors that link the cell to the ECM is important for cell function, spreading and growth. Substrate stiffness can also affect the adhesion of cells, the assembly of the cytoskeleton and cell motility (Discher, Janmey and Wang, 2005), which is investigated further in Sections 4.2.1.2 and 4.2.3 of this Chapter.

4.2.1.2. Quantifying RCE cell attachment to different PDMS blends using the Nikon BioStation CT

Following F-actin staining of RCE cells cultured on different PDMS blends, the number of RCE cells attaching to each PDMS surface and the area coverage of cells across the sample surface were measured using the Nikon BioStation CT, an automated imaging system in which experiments can be performed without having to remove samples from the incubator environment. The methods used are described in Chapter 2, Section 2.6.3.

The F-actin stained RCE cell–PDMS sample images were then analysed using the Nikon CL-Quant automated image analysis software which uses preconfigured standard assays and also allows the user to create analysis assays specific to their data, allowing batch processing of large image data sets. The method of analysis used was a set of procedures customised to the RCE cell–PDMS sample images to ensure the correct measurement of RCE cells on the surface of the PDMS blends. The methods used are described in more detail in Chapter 2, Section 2.6.3.

Figure 40 shows the percentage coverage of RCE cells on the surface of the different PDMS blends following 24 hours in culture, fixation and staining of PDMS–RCE cell samples. Samples were imaged in the BioStation CT and tiling images of the whole well contain a sample were taken at 10X magnification in phase contrast and fluorescence in order to image the whole PDMS surface to further investigate the effectiveness of the PDMS surface properties in promoting cell adhesion and to compare this across the PDMS blends.
Figure 37 – Percentage RCE cell coverage of RCE cells cultured on PDMS blends after 24 hours of culture and fixation. RCE cells were stained with phalloidin (F-actin) and DAPI (nuclei). Images were quantified using CL Quant (Nikon) and the area measured for each well was 9.6 cm² or the whole well in a 6-well plate, covering the whole PDMS sample size of 2 cm² (mean ± SD, n = 3). * shows a statistically significant increase in % cell coverage in plasma treated PDMS 184 compared to untreated PDMS 184 (p ≤ 0.02). ***shows significant increase in % cell coverage after plasma treatment for PDMS 1:1 p ≤ 0.001 (one-way ANOVA using Tukey analysis, Minitab).

The images taken were used to quantify the attachment profiles of the RCE cells on each PDMS blend by using a modified analysis method (Chapter 2, Section 2.6.3) to mask the cell areas using the fluorescence to differentiate the RCE cells from the PDMS in the background. This gave the percentage coverage of RCE cells when subtracted from the total area.

Untreated and plasma treated PDMS – RCE cell samples were compared and it was found that there were significant differences between PDMS 184 when comparing untreated to plasma treated samples and also between 1:1 PDMS samples in terms of percentage cell coverage observed. PDMS 184 showed a significant increase in the percentage RCE cell coverage after plasma treatment from 67.1 ± 17.6 % on untreated PDMS 184 to 98.6 ± 0.3 % on plasma treated PDMS 184, which followed the trend observed in the fluorescent staining images (Figures 35-39). This data further supported the data shown in Figure 21 (Chapter 3) that PDMS 184 was the more successful PDMS blend used in
terms of the uptake of surface modification (Chapter 3, Section 3.2.1.2) and also had increased attachment of RCE cells to the PDMS surface when compared to other PDMS blends. This may have been linked to the stiffness data obtained in Chapter 3 (Section 3.2.2), which showed that in the AFM data, PDMS 184 had the highest Young’s modulus compared to PDMS 10:1, PDMS 5:1 and PDMS 1:1. However, this was not the case for the tensile testing results and ESPI results obtained and PDMS 184 Young’s modulus from these mechanical tests was not significantly different to the other PDMS samples tested (Chapter 3, Figures 31-33).

It was also observed that for PDMS 1:1, there was a significant increase in the percentage RCE cell coverage after plasma treatment from 44.3 ± 17.8 % on untreated PDMS 1:1 to 98.3 ± 0.6 % on plasma treated PDMS 1:1. However, the percentage RCE cell coverage observed for PDMS 1:1 on the untreated PDMS sample was significantly lower than the other PDMS blends measured. This result did not correlate with the trends observed in the surface and bulk mechanical properties (Chapter 3) as PDMS 1:1 had a lower Young’s modulus when compared to PDMS 184, PDMS 10:1 and PDMS 5:1 in two of the three mechanical testing methods used, tensile testing and AFM. However, PDMS 1:1 had the highest Young’s Modulus when compared to PDMS 184, PDMS 10:1 and PDMS 5:1 using the ESPI measurement technique. The stiffness of PDMS 1:1 was also significantly less when compared to PDMS 184 in the AFM results described previously (Chapter 3, Figure 33).

PDMS 1:5 showed lower percentage cell coverage after plasma treatment when compared to the other plasma treated PDMS samples (Figure 40). This was thought to be due to the material properties of PDMS 1:5 as discussed in Chapter 2, Section 2.1.2, supported by the RCE cell – PDMS sample staining images shown for PDMS 1:5 (Figure 39). The morphology of the RCE cells cultured on PDMS 1:5 appeared to be very different from the rest of the PDMS blends, with a more rounded shape and markedly less RCE cell spreading and F-actin visualisation (Figure 39). The results obtained in the work so far are closely linked when PDMS 1:5 was investigated as it did not provide a preferential surface for RCE cell attachment and spreading and this was thought to be strongly related to the bulk mechanical properties and polymer chemistry of PDMS 1:5 as described previously.

Overall, the data obtained in Figure 40 did support key observations in particular regarding the stiffest and softest PDMS blends, PDMS 184 and PDMS 1:5 respectively. It showed that there was an increase in the percentage coverage of RCE cells on all PDMS blends after plasma treatment, which further supported the success of the plasma treatment used. It also highlighted the lower percentage RCE cell coverage for PDMS 1:5 after plasma treatment, which was expected. The methods used required further testing and calibration using several more images in order to
accurately determine the number of RCE cells attached to the whole PDMS surface rather than percentage coverage and to reduce the error in the measurements. Percentage coverage was a good indication and estimation of RCE cell attachment over the surface of the PDMS samples but did not provide an exact number of cells attached to each PDMS sample.

Experiments carried out from this point in the work did not use PDMS 1:5 as a substrate blend. This was decided based on the results obtained for bulk mechanical properties, surface characterisation, actin staining and the percentage RCE cell coverage experiments. PDMS 184, PDMS 10:1, PDMS 5:1 and PDMS 1:1 were still used and this was thought to provide a good range of Young’s moduli for testing with RCE cells.

4.2.2. RCE cell viability and proliferation when cultured on PDMS blends

Following investigations into RCE cell attachment and cytoskeletal changes after 24 hours in culture and quantifying the percentage RCE cell coverage after this period, the viability and the proliferation of the RCE cells cultured on the different PDMS blends was measured. This was done using PrestoBlue® cell viability reagent, which could be used as a live cell assay. The viability and proliferative capacity of the RCE cells was tested using a continuous live culture over 8 days to see the effects of PDMS blends on RCE cells over a longer period of time. The methods used for this assay are described in detail in Chapter 2, Section 2.6.4.

Figure 41 shows the different PDMS blends used and the control culture on tissue culture plastic and the general trend in RCE cell viability over 8 days in culture can be observed.
Figure 38 - A comparison of the average percentage reduction of PrestoBlue® cell viability reagent by RCE cells cultured over 8 days on different plasma treated PDMS blends and on tissue culture plastic (mean ± SD, n=3).
In Figure 41 the overall trend observed in the cell viability of RCE cells over 8 days in culture was that there was an increase in RCE cell viability and culture on the PDMS blends did not have a cytotoxic or detrimental effect on the RCE cell viability. It has been shown in the literature that Sylgard 184 and Sylgard 527 do not have cytotoxic effects on cells and Sylgard 184 in particular has been extensively used in research to produce substrates for cell culture (Palchesko et al., 2012; Dow Corning, 2014a, 2014b; Lycans et al., 2014). However, to test the PDMS samples used in this work with the RCE cell line, viability and proliferation studies were carried out.

Cell viability and proliferation were measured in the assay by the percentage reduction of the PrestoBlue reagent as the metabolising and therefore living RCE cells would reduce the component in PrestoBlue called Resazurin (blue, non-fluorescent), which changed to Resorufin (red, fluorescent). The colour change in the RCE cell culture medium was then detected by fluorescence optical intensity. The tissue culture plastic control showed that up to day 4 in culture, the RCE cells showed a greater increase in viability and proliferation, which indicated that they were in the growth phase, with metabolically active cells. By day 5, cell proliferation rate had slowed and then from days 6 – 8, plateaued by the end of the culture period. This trend was not observed in the PDMS blends shown in Figure 41. Overall, the viability and proliferation of the RCE cells increase over the 8 day culture period but at a much slower rate compared to tissue culture plastic. This was expected due to the change in the culture substrate and also the differences in mechanical and surface properties of the PDMS blends, as discussed previously.

By day 8, the percentage reduction of PrestoBlue by RCE cells was found to be very similar for PDMS 10:1, PDMS 5:1 and PDMS 1:1, at 22.4 %, 24.9 % and 23.6 %. For PDMS 184, the percentage reduction of RCE cells cultured on this substrate and therefore the RCE cell viability was highest by day 8 in culture when compared to the other PDMS blends measured at this time point at 28.9 %. This showed that PDMS 184 was the most appropriate cell culture substrate in terms of RCE cell viability and promoted cell adhesion, proliferation and spreading of the RCE cells on its surface. This was also supported by the data obtained in Chapter 3 for the bulk mechanical properties of the different PDMS blends and is related to the preference of cells to attach to stiffer substrates, which has been reported in the literature (Lo et al., 2000; Discher, Janmey and Wang, 2005; Rehfeldt et al., 2007; Schwarz and Gardel, 2012).

It was observed that PDMS 10:1 had a lower percentage reduction of PrestoBlue overall across the time points after day 5 in culture when compared to the other PDMS blends. PDMS 184 had lower PrestoBlue reduction than all other PDMS blends but by day 6 in culture, had increased percentage reduction by RCE cells over all other PDMS blends for the remaining time points. PDMS 5:1 and 1:1
also had higher PrestoBlue reduction between days 1-5 in culture and then started to decrease between day 5 and 6. Due to the nature of the PrestoBlue data, the exact cell number is not calculated and PrestoBlue reduction by RCE cells is measured by fluorescence using the FLUOstar Omega plate reader. The percentage PrestoBlue reduction is worked out by dividing the fluorescence reading from a sample of a particular PDMS blend seeded with RCE cells by the fluorescence reading for the positive control (100% reduced PrestoBlue reagent in RCE cell culture medium) and multiplying by 100. This was done for all RCE cell – PDMS samples used in the experiment and is described in more detail in Chapter 2, Section 2.6.4.

A PrestoBlue calibration curve for RCE cells was carried out before conducting the experiments and this was then used to predict the number of RCE cells present at each time point. A regression analysis was performed using Minitab and the methods used to derive the predicted cell number have been described in Chapter 2, Section 2.8. The data in Figure 41 was then plotted using the predicted cell numbers to further investigate how the PrestoBlue percentage reduction translated to cell number, as shown in Figure 42.

**Figure 41** – Predicted cell number from the PrestoBlue cell viability assay data showing the estimated cell number calculated using regression analysis. Predicted cell numbers are shown across the PDMS blends and tissue culture plastic over 8 days in culture. Regression analysis performed using Minitab, p value for quadratic model ≤ 0.005.
Figure 42 showed that the estimated RCE cell numbers cultured on PDMS blends were similar on day 1 and were around 40,000 – 50,000 RCE cells, except for PDMS 184 and PDMS 10:1, which were lower and had predicted cell numbers of approximately 27,000 and 35,000 respectively on day 1.

The seeding density used for the these RCE cell - PDMS samples was $8.3 \times 10^3$ RCE cells/ cm$^2$, which was approximately 80,000 RCE cells per PDMS sample. Therefore the predicted cell numbers were closer to the actual seeding densities used on the RCE cell – PDMS sample for the PrestoBlue viability assay. This was due to the methodology used to prepare the RCE cell – PDMS samples for the assay after seeding. To ensure that only RCE cells cultured on the surfaces of the PDMS samples were measured for the 8 day viability and proliferation assay, after RCE cell seeding and 24 hours incubation, the RCE cell – PDMS samples were then removed from the well plate using sterile tweezers and were transferred to a new sterile 6 well plate (see Chapter 2, Section 2.6.4). In doing so, some of the RCE cells would have remained in the original plate that the PDMS sample was seeded in as low attachment plates were not used. This is why there was a reduction in the number of predicted RCE cells at the start of the assay. However, a similar trend in Figure 42 was observed in the RCE cells on TCP and PDMS blends when compared to the trends in Figure 41, as the relative fluorescence (RFU) was used from the PrestoBlue calibration curve to determine estimated cell numbers. When looking at the predicted cell number (Figure 42), all of the PDMS blends showed very similar predicted RCE cell numbers until day 6, where differences between the PDMS blends were more apparent when compared to Figure 41. The predicted RCE cell numbers for PDMS 184 were highest out of all the PDMS blends from day 6 (Figure 42), which was also found when percentage reduction of PrestoBlue was compared (Figure 41). PDMS 5:1 and 1:1 followed the expected trend of decreasing predicted cell numbers but PDMS 10:1 had the lowest predicted RCE cell numbers after day 6 when compared to the other PDMS blends (Figure 42).

Following the PrestoBlue viability assay, it was decided that based on this data the culture period used would be less than 72 hours of RCE cell culture on PDMS samples. This was implemented in further experiments because from the viability experiments, this time period was identified as having less variation in the viability and proliferation of RCE cells cultured on PDMS and in the predicted RCE cell number obtained. Also in the control condition, RCE cells cultured on TCP, the RCE cells were observed to be in the growth phase of the culture period and therefore between day 1 and day 3 (24 and 72 hours) of RCE cell culture was the optimum time to test the RCE cell – PDMS samples.
4.2.3. Investigating the effects of different PDMS substrates on RCE cell migration

Cell migration assays were performed on RCE cell – PDMS samples and on RCE cells cultured on tissue culture plastic as described in Chapter 2, Section 2.7 in more detail. This was done in the form of scratch wound assays on a confluent layer of RCE cells cultured on a PDMS substrate. The confluent RCE cell layer was physically scratched or wounded using a pipette tip and the samples were then imaged using the BioStation CT, an automated imaging system and incubator. Following this the rate of closure of the scratch wound was measured using a customised wound healing recipe available on the Nikon CL-Quant analysis software. For each condition, measurements over 12 hours were used and the change in the wound to image size area ratio were compared to show the change in the percentage wound closure over time.

Figure 40 – The percentage wound closure between 0 to 12 hours in culture following the scratch wound assays on RCE cells cultured on TCP and plasma treated PDMS blends. ** showed a significant decrease in % wound closure of RCE cells on PDMS 1:1 when compared to TCP (p ≤ 0.004). * showed a significant decrease in % wound closure of RCE cells on PDMS 1:1 when compared to PDMS 10:1 (p ≤ 0.05) (mean ± SD, n=3) (One-way ANOVA using Tukey analysis, Minitab).
The percentage scratch wound closure was compared between TCP and PDMS 184, PDMS 10:1, PDMS 5:1 and PDMS 1:1 (Figure 43). In order to standardise the images collected of the scratch wound closure over time, they were limited from 0 to 12 hours for the image analysis, with 0 being the start of the assay following the scratch wound procedure. The percentage wound closure values obtained for the scratch assays were; TCP = 36.4 ± 1.3 %, PDMS 184 = 17.8 ± 11.2 %, PDMS 10:1 = 27.3 ± 11.7 %, PDMS 5:1 = 20.4 ± 3.6 % and PDMS 1:1 = 8 ± 5.8 %.

The overall trend observed in the RCE scratch wound assays was that in the control condition on TCP, the RCE cells had the highest percentage wound closure compared to the PDMS blends. This was expected as the differing Young’s moduli of the PDMS blends have already been shown to impact on the RCE cell structure and adhesion behaviours. PDMS 184 had a lower percentage wound closure rate when compared to the other PDMS blends. This was not expected as PDMS 184 was found to have the highest Young’s modulus compared to the rest of the PDMS blends in Chapter 3 (Figure 33) where the bulk mechanical properties were tested. However this was not the case when other techniques were used such as tensile testing and ESPI (Figures 31 and 32). The lower percentage wound closure rate for PDMS 184 could have been due to other factors such as variation generated by using the scratch wound assay method as a manual scratch was created in the RCE cell layer (Kramer et al., 2013) or the variability observed in the material mechanical properties.

However, the trend observed in Figure 43 for PDMS 10:1 to PDMS 1:1 showed that with decreasing Young’s modulus, the percentage RCE cell wound closure over 12 hours also decreased. A significant decrease was observed for PDMS 1:1 when compared to TCP and PDMS 10:1, and PDMS 1:1 was the softest PDMS blend and had the lowest Young’s modulus. This trend correlated with the change in stiffness of the PDMS substrates and it was apparent that the substrate mechanical properties had an effect on the motility of RCE cells. The data in Figure 43 showed that as the PDMS blend became softer, the motility of the RCE cells decreased and began to inhibit the physical behaviour of the RCE cells. The significant decrease in percentage wound closure of the softest PDMS blend PDMS 1:1 when compared to the control on TCP showed the impact of the lower Young’s modulus on RCE cell migratory behaviour and their response to mechanical changes in their surrounding environment. Although the result for PDMS 10:1 was higher than expected (Figure 43) it was still lower than the control condition of TCP. The results determined that the lower the Young’s modulus of the PDMS substrate, the slower the rate of percentage wound closure when data was compared over a 12 hour period.
4.3. General discussion and conclusions

Overall the results obtained in this Chapter showed that as the stiffness or Young’s modulus of the PDMS blends used decreased, changes in RCE cell attachment and morphology were observed in terms of the F-actin and stress fibre formation and RCE cells cultured on the softest PDMS blends appeared more rounded and less spread onto the PDMS surface. This was thought to be due to the cell-matrix interactions on softer substrates as this contact is critical for anchorage – dependence and other cell functions (Rehfeldt et al., 2007). The adhesion of cells to substrates allows the cell to probe the surrounding ECM and sense the mechanical changes in the surrounding environment. The conversion of the mechanical force sensed by the cell into biochemical signals within the cell is important in several cellular processes and it is the integrin receptors that play a major role in cell adhesion and material interactions with substrates (Fusco et al., 2015). Integrins can either act as mechanotransducers directly transmitting forces from the ECM to the cell or transmitting force to other components. They can also be intermediate receptors in other pathways that stimulate the integrins (Ross et al., 2013). The cell attachment through contractile stress fibres is key for adhesion and maintaining cell attachment. The F-actin staining images showed that as the Young’s modulus of the PDMS substrate decreased, less F-actin and stress fibres were visible, suggesting that the RCE cells were unable to maintain attachments to the softer PDMS substrates. RCE cell attachment was also quantified and showed a decrease in the number of RCE cells attached to the softest PDMS blend PDMS 1:1, supporting the RCE cell F-actin staining images. This method required further optimisation to establish accurate counts of RCE cells attached to the PDMS substrates and more images were required for the analysis to reduce the variability in the data.

The viability and proliferation of the RCE cells cultured on the PDMS blends increased over an 8 day culture period and although the viability was lower than RCE cells cultured on TCP, it showed that overall the PDMS blends did not cause any cytotoxic effects to the RCE cells during the culture period. The predicted cell numbers from the PrestoBlue viability assays were calculated using a regression analysis from the PrestoBlue calibration curve. The viability assays and predicted RCE cell numbers showed that up to 72 hours in culture, RCE cell – PDMS samples were at the optimum growth phase as observed by the viability assay on RCE cells cultured on TCP. The cell numbers were not as variable between the different PDMS blends in the first 3 days of culture whereas after longer culture periods, differences in RCE cell viability were more apparent. Some process modification would be required going forward as there was a reduction in the predicted RCE cell number at the start of the PrestoBlue assay from the seeding density used.
RCE cell response to changes in the substrate stiffness was further investigated by using scratch wound assays and the results showed that as the Young’s modulus decreased from PDMS 10:1 to PDMS 1:1, the percentage wound closure also decreased over a 12 hour period. Although PDMS 184 had a lower percentage wound closure compared to PDMS 10:1, it was also found to have a lower percentage wound closure compared to TCP. This was observed across all PDMS blends when they were compared to TCP. Factors affecting cell migration include changes in substrate stiffness and generally it has been observed that some cell types will migrate towards a stiffer surface (A. Kim et al., 2012). Here the RCE cells did not have a stiffness gradient and so the effects of the different PDMS blends with a range of Young’s modulus could be tested on certain cell behaviours that are known to be affected by substrate stiffness.

The results observed in this Chapter combined with the results in Chapter 3 showed that the PDMS blends produced formed substrates with a range of different Young’s modulus. By culturing these substrates with RCE cells, the physical responses of the RCE cells could be observed in terms of how the macroscale or bulk mechanical properties affected RCE cell behaviour. The key observations were that significant differences could be seen in RCE cells cultured on the softest PDMS blend, PDMS 1:1, which seemed to be distinctly different in most conditions investigated except for the PrestoBlue viability assays. This showed that the lower Young’s modulus had more of an impact on RCE cell adhesion, proliferation and migration.

Following the macroscale studies and the response of RCE cells to the bulk mechanical properties of the PDMS blends, micro- and nano-scale investigations were carried out on RCE cells cultured on the different PDMS blends to investigate the forces in play at the cell sensing level.
Chapter 5
Changes in Substrate Stiffness and the Effects on Cell Mechanics
5.1. Introduction

The substrate used for cell culture can influence not only the morphological and physical properties of a cell but also the mechanical properties of the cell. The mechanical properties of cells are known to be important for several biological processes such as cell proliferation, cell motility or migration, apoptosis and differentiation (Steward et al., 2010; Huang et al., 2012). It is important to further our understanding of how changes in the mechanical properties of the extracellular environment (ECM) can impact on the mechanical properties of a cell. By understanding the mechanical changes that occur at the cellular level, the cell – matrix interactions that are involved in important biological processes such as wound healing can be further investigated (Miron-Mendoza et al., 2013).

In order to study the mechanical changes at the cellular level, Atomic Force Microscopy (AFM) has been used to measure the mechanical properties of cells at nanoscale. This technique provides a non-destructive method to measure the mechanical properties of soft biological material and also allows single cell measurements. AFM can be used to measure the nanoscale forces in real time and also obtain topographical information without damaging the sample (Liu and Sheardown, 2005; Valero et al., 2016).

In the previous Chapters, the bulk mechanical properties and surface modification of Polydimethylsiloxane (PDMS) substrates were analysed to produce a range of different PDMS substrates that were suitable for Rabbit Corneal Epithelial (RCE) culture. To analyse the effects of the PDMS substrate stiffness on RCE cell culture, RCE cell attachment, proliferation and migration were investigated. Four PDMS substrates with varied stiffness were used in this Chapter to further investigate the effects of the different PDMS substrate mechanical properties on the nanoscale properties of the RCE cells and single cell interactions with the PDMS surface. Changes in mechanical properties at the cellular scale were measured to assess the influence of substrate bulk and surface properties on RCE cell mechanical properties and how other factors could impact on cell mechanics.

5.1.1. Aims and Objectives

The aim of this Chapter is to study the effects of PDMS substrate stiffness and surface properties on RCE cells on a single cell level and to investigate how the different substrate mechanical properties of the PDMS blends impact on cellular properties such as Young’s modulus. The objectives to achieve the aim are to measure the Young’s modulus of RCE cells cultured on the surface of different PDMS blends by using AFM. This method will also be used to look at surface roughness of the PDMS samples and RCE cells that are attached to the PDMS surfaces. Specific areas of the RCE cells will be measured and compared over the different PDMS blends in terms of surface roughness and Young’s
modulus to determine whether the varying stiffness of the PDMS substrates affects the mechanical properties of the RCE cells.

5.1.1.1. Hypothesis
It is hypothesised that as the ratio of Sylgard® 527 to Sylgard® 184 increases in the PDMS blends (from PDMS 184 to PDMS 1:1 (Sylgard 184: Sylgard 527)), the PDMS surface will decrease in roughness. It was also hypothesised that there will be a decrease in surface roughness of the RCE cell the further away from the nucleus region of the cell. This was thought to be due to the cell body and cell edge areas being flatter than the nucleus and therefore increasing contact with the PDMS surface. In terms of Young’s modulus of the RCE cell regions, it is hypothesised that there will be a general trend of decreasing Young’s modulus of a specific region of the RCE cells being measured as the Young’s modulus of the PDMS blends decrease (from PDMS 184 to PDMS 1:1). It is thought that from the nucleus to the cell edge of the RCE cells there will be an increase in Young’s modulus overall irrespective of PDMS blend. This is due to the attachment of RCE cells to the PDMS surfaces and the interaction of the RCE cell cytoskeleton, which is expected to display significant differences in Young’s modulus because of an increased effect of the substrate properties on the RCE cells.
5.2. Results

5.2.1. Surface Roughness of different PDMS blends and different regions of the RCE cell measured using AFM and QI™ mode

Surface topography of different PDMS samples, RCE cells attached to the surface of different PDMS samples and RCE cells attached to tissue culture plastic was investigated using AFM. The differences in surface roughness (average roughness Ra) were also compared.

5.2.1.1. Comparing the average roughness of PDMS 184 and PDMS blends 10:1, 5:1 and 1:1

![Figure 41 – Average Roughness Ra of the surface of PDMS 184 and PDMS blends 10:1, 5:1 and 1:1 (mean ± SD, n=3) * shows a significant decrease in surface roughness from PDMS 5:1 to PDMS 1:1 p = ≤ 0.04. (One-way ANOVA using Tukey analysis), Minitab.](image)

The differences in surface roughness (average roughness Ra) were compared across the different blends of PDMS used in experiments, as shown in Figure 44. It was observed that there was a general trend between PDMS 184, 10:1 and 1:1 in that the average roughness Ra of the PDMS samples decreased overall as the amount of PDMS 527 in the blends increased. However it was found that the average roughness Ra for PDMS 5:1 (ratio of Sylgard 184: Sylgard 527) increased
when compared to the other three PDMS samples with an average roughness of 261 nm ± 99.6 nm (Figure 44).

The average roughness of PDMS 1:1 was found to be statistically lower than the roughness of PDMS 5:1 surface (p = 0.04). This could have been due to the ratio of the two components that made up these blends, Sylgard 184 and Sylgard 527. PDMS 1:1 contained equal parts of both types of Sylgard whereas PDMS 5:1 contained 5 parts Sylgard 184 to 1 part Sylgard 527. However this was greater than in PDMS 10:1, which contained 10 parts Sylgard 184 to 1 part Sylgard 527. The varying amounts of Sylgard 527 appeared to have an impact on the surface topography of the resulting PDMS blend, and a significant decrease in average roughness Ra of PDMS between PDMS 5:1 and PDMS 1:1 was observed.

To further investigate the differences observed in surface roughness of PDMS samples, Figure 45 shows a three – dimensional (3D) view of a representative image of each PDMS type giving the height (measured). This equates to the overall peak to valley roughness Rt across the sample, which is the total height of the roughness profile for each sample shown. The differences in surface roughness using the height (measured) can also be seen here and a similar trend is apparent when compared to the average roughness Ra shown in Figure 45 in that the height (measured) for PDMS 5:1 is higher in the images in Figure 45 and Figure 44.

Further image processing carried out on the 3D images in Figure 45 was Gaussian smoothing (width of 0.261 µm X and Y direction). The differences in the 3D images when comparing PDMS 1:1 to the other PDMS types could have been due to the lower stiffness of PDMS 1:1 which may have caused increased lateral deflection of the cantilever during scanning as the cantilever may have been sticking to the PDMS 1:1 surface causing lines or grooves to appear on the scan image. However, for each condition the same setpoint and z-length was used to maintain consistency in the tip-sample separation and to apply the same settings of force to each sample. When calibrating the spring constant of the tip, the tilt angle is considered by the SPM software as this is important in spring constant calibration. However additional correction factors can be used to take into consideration the effects of the measurements in liquid.
Figure 42 – AFM images showing a representative 3D scan image for each PDMS type. Scale bars show the scan size of 50 x 50 µm and the height (measured) of the sample, representing the peak to valley roughness Rt (µm).

5.2.1.2. Comparing the average roughness of the nucleus and cell body of RCE cells attached to different PDMS samples and tissue culture plastic

The average roughness Ra was also measured for RCE cells cultured on the different PDMS samples and tissue culture plastic. The specific areas of the cell that were studied were the nucleus and the cell body. The substrate on which the RCE cell was attached to was also measured for surface topography (see figure 48). Figure 46 compares the average surface roughness of the nuclear and cell body regions of the RCE cell when cultured on different PDMS samples and tissue culture plastic (TCP), as well as the PDMS and TCP surface roughness measurements as controls to compare the substrate topographies to RCE cell topographies.
Figure 43 – Average roughness $Ra$ of RCE cells attached to different PDMS sample surfaces and tissue culture plastic comparing the average roughness $Ra$ of the nucleus, cell body and surrounding substrate (mean ± SD, $n=3$). *** shows significant differences between RCE cell roughness of the nucleus on different PDMS blends and TCP when compared to the cell body of an RCE cell on the same type of PDMS and the substrate. $P$ value for all comparisons ≤ 0.001 (one-way ANOVA using Tukey analysis, Minitab).

It was found that there was an overall trend in average roughness of the selected features of the RCE cell and the roughness of the surface decreased from the nucleus to the cell body. This may have been the result of the cell body and cell edge of the RCE cell becoming thinner and spreading across the surface of the substrate the further away from the nucleus. The setpoint height values have been compared later on in the Chapter.

Statistical analysis showed that there were no significant differences between the different PDMS roughness measurements and the general trend of decreasing roughness between the nucleus and the cell body was observed across all PDMS samples (Figure 46). Differences were found between the roughness of the nucleus and cell body when comparing RCE cells on different types of PDMS and TCP. This supported the observed trend of a significant decrease in roughness from nucleus to cell body, showing that it was present across the different substrates and was a characteristic of the RCE cell.

When nuclei were compared across the different PDMS substrates and TCP, differences were found in average roughness of the nuclei on PDMS 10:1 compared to PDMS 184 and TCP nuclei, showing that on PDMS 10:1, RCE cell nuclei were significantly rougher. The roughness of the nuclei region on
TCP was also significantly lower than the nuclei roughness of RCE cells on PDMS 5:1 (Figure 46). The introduction of the softer Sylgard 527 to Sylgard 184 to produce PDMS blends appeared to have an effect on PDMS surface roughness, which was more obvious for PDMS 5:1 (see Figure 45), and therefore may have impacted on the attachment of the RCE cell to the substrate, altering the cell shape and cell mechanics, lending to the differences observed in nuclei surface roughness (Zamani et al., 2013). However, when comparing the cell body across the different PDMS types, no significant differences were found between the roughness data. This showed that the surface roughness properties of the RCE cell body remained within a similar range across different PDMS substrates including TCP, suggesting that the surface properties of the substrate did not have significant effects on the RCE cell body surface roughness.

For PDMS 184, significant differences were found between the roughness of the PDMS surrounding the cell and the nucleus and also between the cell body and the nucleus of the RCE cell attached to this type of PDMS. This was observed across the four types of PDMS and a similar trend was found. The way in which the RCE cell attached to the PDMS, determined this pattern in surface roughness and it was clear that this was inherent to the RCE cell and not due to the influence of the PDMS.

Comparing RCE cells cultured on TCP, the trend observed previously of a decrease in average roughness between the nucleus and cell body was also found in RCE cells cultured on TCP (Figure 46). The average roughness of the RCE cell nucleus on TCP was found to be significantly higher in comparison to the average roughness of the TCP surface. Significant differences in roughness were also found between the cell body and nucleus on TCP and between the cell body and the TCP surface. No significant differences between the roughness of the RCE cell body and the PDMS or TCP surface surrounding the RCE cell were found, across all samples that were measured. Significant differences were also found between the roughness of the RCE cell body and the PDMS or TCP surface surrounding the RCE cells and this was expected due to the different materials used and the RCE cell cytoskeletal structures that would be measured.

5.2.1.3. Height images of RCE cells on PDMS blends and TCP
3D images of AFM scans carried out on RCE cells attached to PDMS and TCP were further studied to investigate any changes in RCE cell structure and morphology when attached to different substrates. Figure 47 shows representative images of RCE cells cultured on different PDMS samples and on TCP. The overall peak to valley roughness Rt for each sample is shown as the height (measured). In terms of the peak to valley roughness of the RCE cell, the scale shown in the 3D images equates to the maximum height value for the image, which is the nucleus region of the RCE cells, as depicted by the colour scale used. The trend observed in Figure 46 for the average roughness $Ra$ of the nucleus
across all culture substrates can also be seen in Figure 47 as the height (measured) increases from PDMS 184 to PDMS 10:1 and then decreases from PDMS 5:1 to PDMS 1:1 and again to TCP. The height (measured) channel is the read out from a sensor that continuously measures the length of the Z piezo. For rougher surfaces with height features over 100nm or in the micron range, it is suggested that height (measured) is used (JPK Instruments, 2015b). Therefore the height images shown in Figure 47 correlated to the height of the RCE cells relative to the surface of the culture surface. The setpoint height is what displays the height value measured by the Z sensor (height measured) when the set point vertical deflection is reached (JPK Instruments, 2016).

The colour scale shown in Figure 47 identifies the structures of the cell based on their height and to make the visualisation of these structures clearer. The change in colour gradient shows that as expected the nucleus was the highest structure and the height of the RCE cells decreased further away from the nucleus toward the cell edge.
Figure 44 - AFM images showing representative 3D scan images for each PDMS type cultured with RCE cells and tissue culture plastic. Scale bars show the scan size of 50 x 50 µm and the height (measured) of the sample, representing the peak to valley roughness Rt (µm). Colour scale showing the changes in height of the cell structures.
5.2.2. Differences in Young’s modulus of specific areas of the RCE cell samples as measured by AFM

The apparent Young’s modulus of certain regions of the RCE cells was calculated using the QI™ data obtained and by applying the Hertz model (Neumann, 2008) to the force curves (see Chapter 2, Section 2.4 and 2.4.6). This was done on measurements taken from PDMS samples cultured with RCE cells and RCE cells attached to tissue culture plastic. The term apparent Young’s modulus has been used, considering the limitation of the assumptions of the biological materials, as described previously in Chapter 2, Section 2.4.6 to fit the Hertz model. However there are several extensions to the original model that allow a better fit of the force curves and the parameters used that influence the measurements are considered. The extensions for different indenter geometries were parabolic, spherical, conical and four-sided pyramid. The different shape of the indenter leads to different radii of the contact circle and will therefore have an effect on the results calculated. These extensions to the Hertz model and therefore the geometry of the indenter determine the equation to be used in the analysis and can be applied within the JPK data processing software (Neumann, 2008; McKee et al., 2011).

5.2.2.1. Comparison of the apparent Young’s modulus of RCE cells cultured on PDMS 184, PDMS blends 10:1, 5:1 and 1:1 and tissue culture plastic

The apparent Young’s modulus of RCE cells cultured on different PDMS types and on TCP was measured using the Hertz model and extensions of the model to calculate the elastic properties of the RCE cells. As the size, shape and orientation of each RCE cell measured were different, areas of each cell were selected using measurement tools in the JPK data processing software to extract the Young’s modulus data from the region of interest. This has been described in more detail in Chapter 2, Section 2.4.7.

The analysed data was collated for four different areas of the RCE cells, which were identified as nucleus, next to nucleus, cell body and cell edge, as shown by the schematic diagram in Figure 48. This was done by using the corresponding setpoint height image of each scan to select the correct regions on the Young’s modulus image and also to standardise each region to a particular setpoint height range and to remove any outliers in the data or values that were outside of the selected ranges (see Table 6).
Figure 48 – A schematic diagram representing an RCE cell attached to the surface of a PDMS substrate showing the areas of the RCE cell and substrate that were measured using AFM depicted in the diagram by the cantilevers. Diagram not to scale.

Although the same imaging parameters were used for each RCE cell measured, there were inherent biological variations in the RCE cells as the cytoskeletal structure, shape and sub-cellular parts of each cell would have an impact on the Young’s modulus measurements (Nikolaev et al., 2014). Also as the RCE cells were fixed after 24 hours in culture, they may have been variations in the cell cycle stage at which the RCE cells were fixed. Sample fixation was not expected to change the RCE cell structure or shape as a whole, but cross-link and denature the proteins in the RCE cells. Due to the variation in the biological data, the data has been summarised as the distribution in Young’s modulus for each area of the cell measured and compared across all PDMS sample types and TCP (Figures 50 – 54). The data has also been displayed as averages of the Young’s modulus for each substrate and each area of the RCE cell being measured (Figure 49).
The Young’s modulus data obtained showed an overall increase in the stiffness of the RCE cell from the nucleus to the cell edge. This was expected as the edges of the RCE cell were in full contact with the substrate and the presence of the cytoskeletal structures, the actin filaments, intermediate filaments and microtubules were likely to be measured in the cell body and cell edge AFM measurements (Blanchoin et al., 2014). Cell adhesion to the substrate is produced by dynamic protein complexes that are coupled to the extracellular matrix (ECM), responding to the forces sensed by the cell through the cytoskeleton. Integrins are important adhesion receptors that connect the ECM to the cytoskeleton and stimulate cell adhesion and cytoskeletal reorganisation through mechanotransduction (Balcioglu et al., 2015). Substrate stiffness has been shown to have an effect on the adhesion structures, the cytoskeleton assembly and how cells spread (Discher, Janmey and Wang, 2005) and therefore could explain the RCE cell stiffness on certain substrates.

The setpoint height information for each area of the RCE cell showed that the nuclei were higher than the other areas of the cell that were measured. For the next to nucleus area, it was observed that there was a slight decrease in the Young’s modulus across all substrates when compared to the nucleus but the decrease was not statistically significant between the Young’s moduli of these areas. It was expected that there would not be a drastic change in the Young’s modulus of the area next to
the nucleus. This may have also have been due to the distinction between the nucleus and area next to it which was done by using the setpoint height of these features. However the differences in the measurements taken for the nucleus region were most likely to do with the RCE cell properties and less about the interaction with the substrate as the nucleus was not in direct contact with the substrate.

When the next-to-nucleus area was compared to the cell body, there was a significant increase in the stiffness or Young’s modulus of the cell body. This increase in stiffness was found across all PDMS substrates and on TCP and showed that the cell body was interacting with the PDMS or tissue culture plastic surface to attach to the surface and activate the cytoskeletal structures, such as the actin and intermediate filaments, which are involved in many dynamic processes in the cell (Steward et al., 2010). Similarly, there was a significant increase in stiffness between the cell body and the cell edge areas of the RCE cells measured across all PDMS substrates and TCP. Comparing the height values, the cell edge measurements had the lowest range of setpoint height values compared to the other cell structures being observed. Therefore the cell edges were closest to the substrate and were the stiffest part of the RCE cell as expected due to the anchorage and contact of the RCE cell with the substrate. As the cell forms an attachment, they examine the elasticity of the environment as they anchor to their surroundings. A normal cell will exert force to the substrate through adhesion molecules such as integrins and also through the stress fibres and contractile machinery of the cell such as the actin and intermediate filaments, leading to a response through the organisation of the cytoskeleton (Maskarinec et al., 2009; Franck et al., 2011). This showed that the cell edges were the stiffest part of the RCE cell as much of the mechanical interaction between the cell and the substrate was carried out through the cytoskeleton, the key structural components of the cell that occupy the cytoplasm or cell body.

Overall the trend observed in the RCE cells across all substrates was that the Young’s modulus was significantly higher for the cell body and cell edge compared to the nucleus and next to nucleus regions. This showed that the trend was inherent to the RCE cell regardless of the substrate it was cultured on. Some of the standard deviations were high for measurements taken as seen in Figure 49 but such variation in the data was expected as biological samples were being tested and multiple different RCE cells were being compared. However, it was observed that the mechanical properties of the different areas of the RCE cell were significantly affected by changes in the substrate mechanical properties when results from different substrates were compared.

The Young’s modulus of the nuclei cultured on PDMS 184 was significantly higher than the nuclei of RCE cells cultured on PDMS blends 5:1 and PDMS 1:1 and also when compared to TCP (Figure 49).
The Young’s modulus of the next-to-nucleus area of RCE cells cultured on PDMS 184 was also found to be significantly higher than the same region measured on PDMS blends 5:1, 1:1 and also TCP. However for both the nuclei and next to nucleus regions, no significant differences were found between RCE cells cultured on PDMS 184 and PDMS blend 10:1. In Chapter 3 (Figure 33) there was a significant decrease in the Young’s modulus of PDMS 184 and PDMS blend 10:1 measured using AFM but this change in the stiffness did not appear to have any effect on the stiffness of the RCE cell nucleus or next to nucleus regions when cultured on PDMS 10:1. It has been observed in previous Chapters that differences between PDMS blends were more apparent towards the softer substrates with lower Young’s modulus. In Figure 49 the trend observed for the nucleus and next-to-nucleus areas was that with decreasing PDMS stiffness, the Young’s modulus calculated for that area also decreased. In fact in the nucleus and next to nucleus regions, the RCE cells cultured on TCP had the lowest Young’s modulus for these cell regions. The trend of decreasing substrate stiffness and decreasing Young’s modulus was also observed in the cell body region data (Figure 49). However this was not observed in the cell edge condition and there was no obvious trend observed.

There was a significant decrease in the Young’s modulus of the cell body region measured on PDMS blends 10:1, 5:1 and 1:1 (Figure 49) when compared to PDMS 184. Also for PDMS blend 1:1, which was the softest PDMS substrate used as shown in Chapter 3 Figure 33, RCE cells cultured on this blend had significantly lower Young’s moduli for the cell body when compared against all other PDMS substrates and TCP. The cell body on TCP showed a significant increase in stiffness when compared to the softest PDMS blend 1:1. The general trend observed for the nucleus, next to nucleus and cell body regions was that as the stiffness of the PDMS substrate decreased so did the stiffness or Young’s modulus of the RCE cell area being measured. However, for the cell edge, this trend did not apply and there was a significant increase in Young’s modulus of the RCE cell edge when PDMS 184 was compared to PDMS 10:1, PDMS 1:1 and TCP. Also the cell edge of RCE cells cultured on PDMS 10:1 had a significantly higher Young’s modulus than the cell edge of RCE cells cultured on PDMS 5:1 and TCP. It was found that on PDMS 5:1, the Young’s modulus for cell edge was very similar to the cell edge Young’s modulus for PDMS 184. However PDMS 5:1 had a significantly lower Young’s modulus for cell edge when compared to PDMS 1:1, the softest PDMS blend, and TCP.

This change in stiffness of the RCE cell edge over the different substrates suggested that after introducing Sylgard 527 to Sylgard 184 to produce the PDMS blend 10:1, there was a greater impact on the Young’s modulus of the cell edge, increasing the stiffness of the cell edge. PDMS 5:1 had a higher ratio of Sylgard 527 to Sylgard 184 compared to PDMS 10:1 but it was found that the Young’s
modulus of the cell edge decreased significantly from PDMS 10:1 to PDMS 5:1. This may have been due to the ratio of Sylgard 184 to Sylgard 527, how they were mixed and the crosslinking of the polymer chains and this would have affected the mechanical properties of PDMS 5:1 and therefore the cell edge stiffness of the RCE cell.

Each Sylgard type had been mixed according to the manufacturer’s guidelines in order to maintain the stoichiometry of the cross–link reaction in the individual Sylgard types used. They were then blended at differing ratios to produce more stable mixtures but the mixing was done by hand for a certain duration, which could have affected the production of a homogenous mixture. On the other hand, a significant increase in PDMS 5:1 surface roughness was observed (Figure 44) when PDMS samples without RCE cells were measured. This suggests that the change in surface material properties of PDMS 5:1 could have affected the formation of anchorage points and focal adhesions and would affect the organisation of cytoskeletal structures and therefore may have impacted on cell attachment. However this link was not found for the increase in Young’s modulus of the cell edge on PDMS 10:1 and PDMS 1:1 and the surface roughness of PDMS 184, PDMS 10:1 and PDMS 1:1 was not statistically different when compared to each other.

PDMS 1:1 showed a significant increase in the Young’s modulus when compared to PDMS 5:1 for cell edge measurements and was the highest Young’s modulus value across all substrates at 232.54 kPa. It was significantly higher than the Young’s modulus of RCE cell edge on PDMS 184, PDMS 5:1 and TCP but there was no significant difference between the Young’s modulus for RCE cell edge on PDMS 10:1 and PDMS 1:1. Therefore the data for cell edge stiffness did not follow the expected trend. It has been shown that the stiffness of the PDMS blends without RCE cells attached did decrease with increasing ratio of Sylgard 527 in the PDMS blend (Chapter 3, Figure 33). Therefore, the PDMS blends were prepared correctly and followed the expected trend due to increasing amounts of Sylgard 527, which produces a PDMS with a very low Young’s modulus as the polymer chains are cross–linked but without the silica nanoparticles that are present in sylgard 184 that stiffen the PDMS (Oláh, Hillborg and Vancso, 2005; Palchesko et al., 2012). It is clear from this data that the RCE cells responded differently to certain PDMS blends at the cell edge, in particular PDMS 10:1 and PDMS 1:1 and that this was indeed the effect of the change in mechanical properties of these specific PDMS blends. The different reactions of the cell edge could be explained by the material properties of the PDMS blends and the way the RCE cells attached to the PDMS surface. The densities of each type of Sylgard and the kit components do differ slightly (Table 5) but it is not a big enough difference for them to be immiscible and so a homogenous mixture could be produced for each PDMS blend.
Table 5 – Table to show the different densities of the kit components for Sylgard 184 and 527.

<table>
<thead>
<tr>
<th>Sylgard type and component</th>
<th>Density (kg/m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sylgard 184 Base</td>
<td>1.11</td>
</tr>
<tr>
<td>Sylgard 184 curing agent</td>
<td>1.03</td>
</tr>
<tr>
<td>Sylgard 527 part A</td>
<td>0.97</td>
</tr>
<tr>
<td>Sylgard 527 part B</td>
<td>0.972</td>
</tr>
</tbody>
</table>

TCP would be expected to be the stiffest substrate used for RCE cell culture with a stiffness value of around 1 GPa (Kolahi et al., 2012; Achterberg et al., 2014). This would be approximately five orders of magnitude higher than the Young’s modulus values found in the substrate only AFM experiments (Chapter 3, Figure 33) that were in MPa for PDMS substrates and six orders of magnitude higher than the areas of the RCE cells that were analysed, which were in kPa. For RCE cells cultured on TCP, the cell edge Young’s modulus was significantly higher than the Young’s modulus of the RCE cell edge cultured on PDMS 184 and PDMS 5:1. Although the TCP Young’s modulus of the cell edge was also found to be significantly lower than on the other two PDMS blends, PDMS 10:1 and PDMS 1:1.

5.2.2.2. Histogram distribution of Young’s modulus data for RCE cell regions

Figures 50 – 54 below show the distribution of the Young’s modulus data extracted from the QI™ processed data files. The histograms show the distribution of the data for four areas of the RCE cell; nucleus, next to nucleus, cell body and cell edge cultured on a specific PDMS substrate or TCP. The data was standardised to the setpoint height ranges given below in Table 6.

The setpoint height images corresponding to a representative RCE cell cultured on a particular substrate and the optical image showing the cantilever of that particular RCE cell are also shown in Figures 50 – 54 E. The colour scale shown next to the setpoint height image (Figures 50-54, F) displays the maximum and minimum colour scale range for the corresponding data for the image and the height ranges shown in Table 6 were used within the maximum and minimum setpoint height to select relevant Young’s modulus measurements for analysis and ignore outliers in the data that were outside of the height ranges.
Figure 50 – Young’s modulus distribution of RCE cells cultured on PDMS 184 across four areas of each cell (images A-D). Images E and F show representative optical and setpoint height images of the same RCE cell showing the cantilever (E) and the AFM height map of the RCE cell (F) n numbers displayed on histograms.
Figure 51: Young’s modulus distribution of RCE cells cultured on PDMS 10:1 across four areas of each cell (images A-D). Images E and F show representative optical and setpoint height images of the same RCE cell showing the cantilever (E) and the AFM height map of the RCE cell (F) n numbers displayed on histograms.
Figure 52 - Young's modulus distribution of RCE cells cultured on PDMS 5:1 across four areas of each cell (images A-D). Images E and F show representative optical and setpoint height images of the same RCE cell showing the cantilever (E) and the AFM height map of the RCE cell (F) with numbers displayed on histograms.
Figure S3 - Young’s modulus distribution of RCE cells cultured on PDMS 1:1 across four areas of each cell (images A-D). Images E and F show representative optical and setpoint height images of the same RCE cell showing the cantilever (E) and the AFM height map of the RCE cell (F) n numbers displayed on histograms.
Figure S4 - Young’s modulus distribution of RCE cells cultured on tissue culture plastic across four areas of each cell (images A-D). Images E and F show representative optical and setpoint height images of the same RCE cell showing the cantilever (E) and the AFM height map of the RCE cell (F) n numbers displayed on histograms.
Table 6 – Setpoint height ranges used to compare Young’s modulus data across four different areas of the RCE cell cultured on different PDMS substrates and tissue culture plastic.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Setpoint height nucleus (µm)</th>
<th>Setpoint height next-to-nucleus (µm)</th>
<th>Setpoint height cell body (µm)</th>
<th>Setpoint height cell edge (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS 184</td>
<td>12-14</td>
<td>10-11.5</td>
<td>8-10</td>
<td>8-10</td>
</tr>
<tr>
<td>PDMS 10:1</td>
<td>12.5-15</td>
<td>10-12</td>
<td>8-10</td>
<td>8-10</td>
</tr>
<tr>
<td>PDMS 5:1</td>
<td>12.5-15</td>
<td>10.5-12</td>
<td>8-10</td>
<td>8-10</td>
</tr>
<tr>
<td>PDMS 1:1</td>
<td>12-14.5</td>
<td>10-12</td>
<td>7.5-10</td>
<td>7-9.5</td>
</tr>
<tr>
<td>Tissue culture plastic</td>
<td>11-14</td>
<td>9-11.5</td>
<td>7.5-10</td>
<td>7-9.5</td>
</tr>
</tbody>
</table>

Using these height ranges, the Young’s modulus was selected and any outliers were not included in the histogram data. Overall, the distribution of the Young’s modulus data for all four areas of interest on the RCE cells improved as the PDMS substrate became less stiff. The setpoint height also played a role in this because at higher heights the distribution of the Young’s modulus data was less normal and there seemed to be more variation such as for PDMS 10:1 (Figure 51 adjusted, Figure 55 below shows data without using the setpoint height ranges). PDMS 10:1 height ranges in Table 6 also show the slight differences in the setpoint height for certain cell areas and this was due to the increased overall height of structures and had to be altered to accommodate the correct areas of the RCE cell being measured. This could have also been due to differences in thickness of the PDMS samples. For PDMS 184 and PDMS 10:1 the distribution of stiffness data was heterogeneous and this correlated with higher structures overall on these PDMS blends when compared to the other PDMS blends and TCP, as shown in Figures 10 and 11. From PDMS 5:1 to PDMS 1:1 the maximum setpoint height range decreases compared to PDMS 10:1 and the effects of this limitation of the data to the particular height range for PDMS 5:1 and 1:1 on the distribution of the Young’s modulus data can be seen in Figures 52 and 53, observing a more homogenous distribution with less lateral variation. For TCP, the Young’s modulus data was better distributed and the setpoint height range was lower than that of PDMS 184, PDMS 10:1 and PDMS 5:1. For the cell edge, the Young’s modulus data became more normally distributed for PDMS 1:1 and TCP and less lateral variation. This showed that on these substrates, the setpoint height also affected the analysis of the RCE cell edge stiffness and improved the quality of the data due to reduction in the setpoint height and therefore less variation in the results for stiffness of the cell. For TCP as it was the most homogenous surface for cell culture than the PDMS blends, this was expected as it would be a more stable surface and would not impact
on the cell edge stiffness data distribution as much as some of the PDMS blends (Figure 54). However, for PDMS 1:1, the softest blend of PDMS used (Chapter 3, Figure 33) the distribution of the Young’s modulus data on the cell edge showed that the RCE cell interaction with the PDMS 1:1 surface was more homogenous than when compared to the stiffer PDMS blends (Figure 53).
Figure 55 - Young’s modulus distribution of RCE cells cultured on PDMS 10:1 across four areas of each cell (images A-D) without using setpoint height ranges for data restriction. Images E and F show representative optical and setpoint height images of the same RCE cell showing the cantilever (E) and the AFM height map of the RCE cell (F) n = 36.
Figures 50 – 54 showed the distribution of the Young’s modulus data obtained for the different RCE cell areas cultured on different PDMS substrates and TCP. The influence of the setpoint height on the distribution of the data for each of the four areas of interest was also tested using the setpoint height range to restrict the data range and the effects of not using this method on the distribution of the Young’s modulus data was shown in Figure 55 in reference to PDMS blend 10:1. The biological variation was inherent to each RCE cell and across different regions and could not be avoided but the data selection was rationalised as best as possible in order to limit some of the variation in the results. At higher structures, the data distribution was affected more and these structures would be the nucleus and next to nucleus regions that would be influenced less by the substrate. However it was also shown in Figure 46 that the nucleus surface roughness was highest across all substrates compared to the cell body, which may have had some influence on the variability in the Young’s modulus data. At lower structures such as the cell body and cell edge of the RCE cells, the substrate had more of an effect on the Young’s modulus but also showed that at lower heights, the data was more normally distributed. This suggested that for PDMS 1:1, the Young’s modulus data for the cell edge was more indicative of what the RCE cell was sensing of the substrate and the response of the RCE cell edge was more reliable due to the distribution of the data and the correlation with the lower setpoint height range observed for all the areas of the RCE cell measured on PDMS 1:1.
5.3. General Discussion and Conclusions

The overall trends observed in the roughness data and Young’s modulus data were that roughness was found to decrease further away from the nucleus and the Young’s modulus increased further away from the nucleus and was highest at the RCE cell edges. There was a relationship between the setpoint height of the sample and the distribution of the Young’s modulus data and RCE cell culture on PDMS 10:1 and PDMS 1:1 showed a significant increase in the stiffness at the cell edge.

The two Sylgard kits used were made of the same material polydimethylsiloxane but Sylgard 184 contained silica nanoparticles which produce a stiffer elastomer when the polymer cross-links (Palchesko et al., 2012; Dow Corning, 2016a, 2016b). Therefore, adding increasing amounts of Sylgard 527 produced a softer elastomer (Chapter 3, Figure 33) and at PDMS blend 5:1 the significant increase in surface roughness may have been due to the change in stiffness of the PDMS. Significant changes in the surface properties of PDMS 5:1 may have been caused by PDMS oligomers from the bulk of the PDMS moving to the surface and creating a layer on the surface of the PDMS, as PDMS 527 did not contain reinforcing nanoparticles and therefore non-cross-linked polymer could affect the surface topography and roughness, leading to lower cell adhesion (Palchesko et al., 2012; Liu et al., 2015).

Other factors to consider are in PDMS 1:1, the 50/50 ratio of each type of Sylgard could have allowed the two types of PDMS to mix properly and maintain stoichiometry of both parts and the final product without leaving any free polymer that had not cross-linked. Further investigation into the mixing of the PDMS blends, curing time and temperature would be required to fully understand the bulk properties of the PDMS blends. Also swelling of the elastomer could play a role in the resulting stiffness of material and as room temperature curing was used for 48 hours per sample, some polymer may continue to cross-link after the set time. However, the PDMS samples were always prepared at least 3 days in advance to allow the full curing time and extra time to ensure the full curing time was met. The RCE cell – PDMS samples as well as the RCE cells on TCP were fixed and stored at 4 °C in PBS until required.

The roughness of the PDMS 1:1 surface without RCE cells was lower when compared to the other PDMS substrates (Figure 44) but the Young’s modulus of the RCE cell edge cultured on the PDMS 1:1 surface was higher than RCE cells cultured on the other PDMS blends (Figure 49). In the same way, when the roughness of the surface of PDMS 5:1 was higher compared to the other PDMS blends (Figure 44), the Young’s modulus of the RCE cell edge cultured on PDMS 5:1 was the lowest when compared to the other PDMS blends and TCP (Figure 49). This suggested that there could have been a correlation between the roughness of the PDMS surface and the Young’s modulus of the RCE cell.
edge that would have the most contact with the substrate surface and therefore be affected more by changes in surface material and bulk mechanical properties of the PDMS blends. However, the idea that surface roughness can improve cell adhesion and affect cell properties may not be the case in some cells for example, for cornea implants a smooth surface topography was more favourable for enhancing the cell adhesion and proliferation as well as cell migration (Raghunathan et al., 2013; Zhou et al., 2016). However for RCE cells cultured on PDMS 5:1 and 1:1 there were differences in the roughness and Young’s modulus data for the RCE cell edge, that indicated the cell edge was affected by the stiffness and surface properties associated with these PDMS substrates.

The results suggested that for a lower surface roughness, adhesion on a PDMS substrate was enhanced due to the increased stiffness of the RCE cell edge. The improved cell – matrix interactions correlated to cytoskeletal contractility whilst the cell was sensing mechanical changes in its environment. The findings suggest that on the softest PDMS substrate PDMS 1:1, the RCE cell had to exert a greater force onto the softer PDMS substrate compared to the stiffer PDMS substrates or tissue culture plastic in order to form cell attachments to the substrate through the cytoskeleton machinery and stress fibres were perhaps more apparent in these RCE cells, hence the stiffer cell edge. In both quiescent and migrating cells, the cell-matrix adhesions tend to assemble initially at protruding cell edges and adhesion to the ECM is started through the lamellipodia and filopodia (Ridley, 2003; Schwarz and Gardel, 2012). This could explain the stiffer cell edges overall and the behaviours of the RCE cells on the PDMS blend surfaces or on TCP in terms of cell migration and spreading following adhesion.

The other areas of the RCE cells and their measured stiffness could have been affected by different cytoskeletal structures present in the RCE cell body, cell nucleus and next to the nucleus. Actin filaments are essential for cell movement and it is the polymerisation of actin filaments that drives the motion of cells (Pollard and Cooper, 2009). The myosin motor proteins are also involved with the actin fibres to generate contractility within the cells and microtubules, another component of the cytoskeleton interacts with the myosin in the communication of cell shape changes and cell migration (Pollard and Cooper, 2009; Huber et al., 2015). It has been shown that the intermediate filament networks are found around the nucleus or at the trailing end of a cell that is actively migrating (Leduc and Etienne-Manneville, 2015). The varied findings from the Young’s modulus results on the different areas of the RCE cell could have been linked to these cytoskeletal structures that have a strong influence of cell behaviour and cell mechanics.
In conclusion this Chapter showed the microscale and nanoscale mechanical properties of RCE cells cultured on the different PDMS substrates and how the stiffness of the PDMS impacted the apparent stiffness of the cell region measured.

Having investigated the microscale and nanoscale mechanosensing of RCE cells on substrates of different stiffness, the next Chapter goes on to further investigate mechanical stimulation on RCE cell – PDMS samples by exerting an external mechanical force on the samples to assess the global cell migratory behaviours of RCE cells in response to stress.
Chapter 6
Mechanical Stimulation of Cells with Uniaxial Stretching
6.1. Introduction

Mechanical stimulation is known to affect cell fate, morphology, orientation, migration and differentiation, which are processes that are crucial to tissue development, function and healing (Kreutzer et al., 2014; Livne, Bouchbinder and Geiger, 2014). There are many commercial and custom-made stretching devices that have been built to apply physical stimulation to different cell types. Several different types of approaches to mechanically stimulating cells have also been developed such as flow-induced shear forces, hydrostatic pressure, substrate stiffness and topography, substrate stretching and indentation of cells (Huang and Nguyen, 2013; Kreutzer et al., 2014).

Although these devices have different advantages and disadvantages, it is challenging to build a device that uses minimal consumption of cells and reagents, that is accurate and consistent in the application of the stimulus, is high throughput and able to perform real-time imaging of the effects of mechanical stimulation on the cells (Huang and Nguyen, 2013). The micro-environment of the cell is influenced by mechanical cues in vivo and this has an impact on the structure and function of living tissue. Mechanical stimulation can induce physiological changes within the extracellular matrix (ECM) of the cell which leads to reorganisation of the cell structure, sensed through focal adhesions of the cell and attachment to the ECM structural components in vivo and the substrate in vitro (Li et al., 2014).

In previous Chapters, PDMS substrates were tested following surface modification for RCE cell culture, attachment, proliferation and migration. The effects of PDMS substrates of differing stiffness on RCE cell processes and the effects of substrate stiffness on the mechanical properties of the RCE cells was further investigated using nanoscale techniques to measure the response at a cellular level. These measurements allowed further insight into the physical and morphological changes that occurred in the RCE cells as a response to different cell culture substrate stiffness. They also allowed nanoscale investigation into single RCE cell interactions with different PDMS substrates and how changes in the cell microenvironment affected the mechanical properties of RCE cells. In Chapter 6, a custom-made uniaxial stretching device was made to perform stretching experiments on RCE cells cultured on a PDMS substrate to investigate the effects of an external mechanical stimulation on RCE cells on a stretchable PDMS elastomer. Changes in the morphology, orientation and position of the RCE cells following stimulation were observed using live cell fluorescent imaging.

6.1.1. Aims and Objectives

The aim of this Chapter is to investigate the effects of mechanical stimulation on RCE cells cultured on PDMS substrates by uniaxial stretching. This will allow further investigation into the impact of
stretching on the RCE cells in terms of cell morphology and orientation on live RCE cells and to see if there is any correlation with the differing substrate stiffness’s of the PDMS substrates. The objectives to achieve the aim are to use a custom-made uniaxial stretching device to perform mechanical stimulation tests on PDMS-RCE cell samples and to observe any changes in RCE cells after stretching using fluorescent microscopy on live RCE cells. Different strain regimes will be used on the PDMS-RCE cell samples to see what impact, if any, this will have on the RCE cells. This will be done by adjusting the percentage stretch or displacement of the PDMS-RCE cell samples and fluorescent live cell imaging of the RCE cells will be carried out before and after stretch.

6.1.1.1. Hypothesis
It is hypothesised that following stretching within the custom-made device, the RCE cells attached to the surface of the PDMS sample will be affected by the mechanical stimulation and will change their shape and positioning in the direction of the stretch. It is thought that this will be more visible with increasing displacement of the PDMS sample. This is due to the uniaxial nature of the stretch, which will be static stretch for a particular time period.
6.2. Materials and Methods

6.2.1. Design and manufacture of a mechanical stimulation device

A mechanical stimulation device was designed and manufactured to apply different strains to PDMS and RCE cell samples and to allow the changes in cell morphology and movement to be monitored via optical imaging before and after strain. The device was designed for offline imaging of live RCE cells as this was an easier, more feasible design for initial experiments and to be constructed in the available time frame. The product specifications are described below, from the perspective of the user who was not involved in the manufacture of the device. Manufacture of the device was carried out by Robert Smith and Michael Ison from the mechanical services workshop, Wolfson School of Mechanical, Electrical and Manufacturing Engineering, Loughborough University.

6.2.1.1. Product Specifications for the mechanical stimulation device

6.2.1.1.a. Performance

- The device should be operated by an electromechanical system and this system should be programmable to fit the required strain regimes relating to the extent of deformation of the PDMS sample. Adjustable deformation should be incorporated into the device and programming to allow a change in the strain at a rate of 1 Hz for cyclic stretching.
- The device as a whole should be easy to use and also have a straightforward assembly and disassembly procedure.
- This assembly and disassembly would be carried out within a biological safety cabinet (BSC) as biological samples were handled so the base plate of the device should be easy to clean with 70% industrial methylated spirits (IMS).
- The working distance for the 10X objective is 15.2 mm which is the objective lens to be used for RCE cell imaging before and after stretch. This would be a consideration regarding the plate used for imaging PDMS-RCE cell samples off-line before and after stretching. If the combined thickness of the PDMS-RCE cell sample and the plate thickness were greater that the working distance for 10X objective, this would affect the ability to image the RCE cell layer on the surface of the PDMS as an inverted microscope was used.
- Nunc™ OmniTray™ 1 well plates were used to contain the PDMS – RCE cell samples. These plates were made from clear polystyrene with good optical clarity for imaging. They are also the correct fit for the microscope stage to be used (Nikon Eclipse Ti microscope). The PDMS – RCE cell samples had to be transferred to these plates for imaging after stretching as the device was designed for off-line imaging.
A custom-made cover for the device would be required to enclose the device parts and PDMS samples so that they are suitable for use within an incubator at 37 °C.

### 6.2.1.1.b. Materials

- The parts of the device that were detachable from the base plate to make them easier to clean had to be made of material that could withstand heat sterilisation at 121 °C and also be water and corrosion resistant.
- The material should also be able to withstand cleaning using 70 % IMS for example soaking in IMS overnight.
- The materials used should be inexpensive and readily available.

### 6.2.1.1.c. Sterilisation and working environment

- The components of the device that come into contact with PDMS – RCE cell samples had to be cleaned by soaking in IMS overnight and allowing the IMS to evaporate in a BSC. These were all parts associated with the clamps.
- The larger parts of the device connecting the clamps to the stationary end and the moving arm of the stretching device were made out of aluminium and were heat sterilised at 121 °C for 15 minutes.
- The outer cover for the device was wiped down before and after each run using 70 % IMS.
- The 1 well tissue culture plastic plates (Nunc™ OmniTray™, Fisher Scientific, UK) were used as disposable containers.
- In order to protect the electromechanical system, during stretching, the device was kept in an incubator at 37 °C but without humidity or CO₂ to prevent any moisture getting into these parts of the device.

Following these specifications, the mechanical stimulation device that was built in house is shown in Figure 56 and the setup within the incubator. Also a schematic drawing of the device setup is shown in Figure 57. Further details on the device are given in this Chapter as well as the preliminary results obtained.
Figure 56 - Mechanical stimulation device showing the setup of the PDMS-RCE cell samples and the device within the incubator.
Figure 57 – A schematic drawing of a bird’s eye view of the final device rig set up using bulldog clips to connect the PDMS-RCE cell samples to the device. Drawing is not to scale.
6.2.2. Linear actuator specifications and calibration of the device

6.2.2.1. Type of linear actuator used to enable uniaxial stretch motion

The uniaxial stretch was performed by a programmable linear actuator and moveable rod. The actuator used was the Firgelli (now Actuonix) L12 – P micro linear actuator with feedback (Actuonix, RS Components Ltd, UK). This linear actuator was used as it was easily available to the user and was a compact, flexible and configurable actuator with the option to use an on-board microcontroller. The L12 actuator is an axial design that has a rectangular cross section, increasing the rigidity of the design (Actuonix Motion Devices, 2016). The linear actuator and other components of the device can be seen in Figure 58.

![Figure 58 - Mechanical stimulation device before sample setup.](image)
The linear actuator used in the device was programmed using an Arduino Nano, which is a small, complete and breadboard–friendly board based on the ATmega328 micro-controller. Arduino is an open-source electronics platform that is based on hardware and software that is easy to use even for people without a background in electronics and programming. The Arduino board can receive input from many sensors and responds by controlling lights, motors and other actuators. To programme the Arduino board, instructions are sent to the microcontroller on the board to tell it what to do. This is done by writing code in the Arduino programming language in the Arduino software and uploading it to the Arduino board.

The linear actuator position meant that by moving the rod forward, the PDMS – RCE sample would be stretched in a uniaxial motion. The Arduino programme was able to adjust the amount of strain exerted on the sample by stretching the PDMS by a certain length. This was translated into the Arduino programme as time in milliseconds. The duration needed to move the rod to a certain length was adjusted for different strains and then held in that position by delaying the programme for a static stretch. The linear guide highlighted in Figure 58 was used to keep the movement of the rod as straight as possible. The operating voltage of the Arduino nano was 5 V and the turning of the motor was controlled through the wiring of the linear actuator to the Arduino board.

When voltage was applied to the motor power pins, the actuator extended and when the polarity was reversed, the actuator retracted. The linear actuator was used as a linear servo as the actuator was connected to the Arduino nano, which was an external controller. The type of linear actuator used had the option of potentiometer position feedback whereby the control board reads the position signal from the actuator, compares this signal with the input control signal and then commands the actuator to move (Actuonix Motion Devices, 2016).

6.2.2.2. Calibration of device and strain protocol for PDMS – RCE cell samples

The mechanical stimulation device was calibrated by using a PDMS sample without RCE cells attached and the strain regimes were tested by measuring the change in length or the deformation of the PDMS sample during stretching, as shown in Table 7. This was carried out to ensure that the input signals to the mechanical stimulation device and the distance that the rod moved did stretch the PDMS by the correct length to exert the correct amount of strain required on the samples.

The calibration PDMS sample size as a whole was 4.5 cm x 4 cm (L x W) and this also had a smaller PDMS insert on top of it placed centrally (see Figures 56, 57 and 60a) and was approximately 4 cm$^2$. The thickness of the PDMS samples used ranged from 600-800 µm. This sample size was then used in all further device runs and the preparation of PDMS samples and further modifications made to prepare them for RCE cell seeding are described in more detail below in Section 6.2.3. The clamps on
each end of the PDMS sample used only during preparation of PDMS-RCE cell samples (autoclaving PDMS and RCE cell culture) covered approximately 1 cm of each end of the PDMS, leaving 2.5 cm of PDMS in between the clamps (Figure 59). The clamps were used only during the autoclaving and RCE cell culture stages of the process because they supported the PDMS sample and kept it from folding over or moving during autoclaving and allowed easier handling of the PDMS samples using sterile tweezers. The clamps were not used in the final assembly of PDMS-RCE cell samples into the device as the bulldog clips shown in Figures 56, 57 and 58 were found to be a much more effective method and easier to assemble and disassemble. However the same lengths were maintained for sample setup within bulldog clips as were used in the clamps.

The change in the length of PDMS in between the clamps was used as a measure for the strain applied to the PDMS. Correlating the displacement of the PDMS with the distance the rod moved allowed the different strain measurements to be calibrated. The different strains applied to the PDMS samples were 10%, 20% and 30% strain, which corresponded to the movement of the rod by 0.25 cm, 0.5 cm and 0.75 cm respectively. The movement of the rod was then cross checked with the change in length of the PDMS sample between the clamps and bulldog clips (Table 7) to ensure that this translated to the displacement of the PDMS.
Figure 59 – A representative diagram of the PDMS 184 sample and PDMS 184 insert arranged in the clamps before any further treatment. These clamps were changed to bulldog clips for assembly into the device. Drawing is not to scale.

Table 7 – The strain applied to PDMS samples and the approximate change in length or displacement of PDMS as a result of the different strains.

<table>
<thead>
<tr>
<th>Strain (%)</th>
<th>Starting length PDMS (between clamps/bulldog clips) (cm)</th>
<th>Distance rod moves and PDMS stretches (cm)</th>
<th>Final length PDMS (between clamps/bulldog clips) (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.5</td>
<td>0.25</td>
<td>2.75</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>2.5</td>
<td>0.75</td>
<td>3.25</td>
</tr>
</tbody>
</table>
6.2.3. Polydimethylsiloxane sample preparation for mechanical stimulation of Rabbit Corneal Epithelial cells

Polydimethylsiloxane (PDMS) was prepared as previously described in Chapter 2, Section 2.1. Sylgard® 184 was used to prepare PDMS 184 for the mechanical stimulation experiments following the methods described in Chapter 2, Section 2.1.1 and was cured for 48 hours at room temperature. In addition to using 100 mm diameter petri dishes for PDMS 184 mixing and curing, smaller petri dishes with a diameter of 35 mm were used to cast PDMS 184 to produce the correct size of well insert for the PDMS samples. 2 g of PDMS 184 was poured into a smaller petri dish, covered and also left to cure for 48 hours at room temperature. The thickness of each PDMS well insert was approximately 0.5 mm and the same amount of uncured PDMS was used each time in the smaller petri dishes. This thickness determined the volume of cell culture medium that could be contained in the well insert, which was 300 µl.

The cured PDMS 184 sections were then cut into the correct size for the mechanical stimulation device using a sterile scalpel. In order to fit into the clamps correctly, the base PDMS section was cut to 4.5 cm x 4 cm (L x W). The PDMS 184 well insert was cut to approximately 2 cm x 2 cm (L x W) or 4 cm² and a small square well was cut out of the centre of the 4 cm² insert, which was approximately 1 cm² in size. This was where the RCE cells would be seeded and the custom made well would contain 300 µl of RCE cell complete culture medium. Using a 1 well tissue culture plastic plate (Nunc™ OmniTray™, Fisher Scientific, UK), the PDMS sample was assembled to be fixed into the clamps (used only for autoclaving and RCE cell culture of PDMS samples), as described in Sections 6.2.2.2 and 6.2.3.2.

Firstly the larger section of PDMS 184 was placed in the 1 well plate using tweezers. The well insert prepared from PDMS was then placed onto the larger PDMS section in the centre and gently pressed down with the tweezers so that the two PDMS 184 sections would stick. Once they had adhered to one another, there was no need to re-stick or use any other adhesives. The PDMS sample was then assembled into the aluminium clamps and each end was clamped to hold the PDMS sample in place for the next few stages of preparation. The base part of the clamp was first placed in the plate and the PDMS 184 sample was lifted to sit on the base of the clamps with one on each end of the sample. Each top part of the clamp was then placed on to the PDMS sample and lined up on each base plate of the clamp. Once aligned, the clamps were then screwed tight to clamp the PDMS sample between both parts and to hold it in place. Figure 60 shows the PDMS sample within the clamps and the arrangement of the parts and a representative diagram of the side view of the clamps to show the position of the PDMS.
Figure 60 – PDMS sample showing the PDMS well insert and clamps in a 1 well plate prior to plasma treatment (A) and a representative side view of the clamping mechanism and how the PDMS was clamped into place for sample preparation, not to scale (B).
6.2.3.1. Plasma treatment of PDMS samples for the mechanical stimulation device

The PDMS 184 samples to be used in the device were then plasma treated to modify the surface of the PDMS for RCE cell attachment. The method used for plasma treatment described previously in Chapter 2, Section 2.2 was followed. The PDMS samples for the device were kept in the aluminium clamps for this part of the process and in a 1 well plate with the lid off whilst being plasma treated. The same programmable XY-translation stage was used to ensure a repeatable and even treatment over the samples. The size of the grid pattern over the surface of the PDMS sample was adjusted to cover the size of the sample but all other parameters were kept the same such as the division width within the grid pattern, gas flow and concentrations used and the same jet was used as previously described in Chapter 2, Section 2.2.

6.2.3.2. Sterilisation of plasma treated PDMS samples for the mechanical stimulation device in preparation for RCE cell seeding

Immediately after plasma treatment, PDMS samples were transferred into a large neck Duran bottle containing pure water and immersed. The clamps were used to keep the PDMS samples separate in the Duran bottle and during transportation to be autoclaved. The Duran bottle containing the PDMS samples mounted on the aluminium clamps was autoclaved on a liquid cycle at 121 °C for 1 hour in order to heat sterilise the plasma treated PDMS for RCE cell culture. Following heat sterilisation, the PDMS samples were then handled in a biological safety cabinet (BSC) and removed from the Duran bottle using sterile tweezers. Each PDMS sample was carefully lifted out of the Duran bottle by holding the clamps and placed in a sterile 1 well plate with the well insert side facing upwards. The samples in their individual plates were then placed on a UV cycle within the BSC for 1 hour. This also allowed any remaining pure water left on the sample after autoclaving to evaporate. After this the samples were then ready for RCE cell seeding and were now sterile.

6.2.3.3. RCE cell staining with CellTracker™ and PDMS seeding

The PDMS sample to be used in the device was seeded with RCE cells that were stained with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes by Life Technologies, Fisher Scientific UK). CellTracker™ is a fluorescent dye that can be used to stain living cells and is well suited for tracking cell movement. It has been designed to pass across the cell membrane and once it is loaded in the cell it becomes impermeable and fluorescent. It is well retained in the cell and can remain over several generations, which is useful for monitoring cells over time. It is non–toxic to the cell and stable and the fluorescence can be detected at 492 nm excitation 517 nm emission and appears as green fluorescence. A stock solution of CellTracker™ Green CMFDA was prepared by dissolving the lyophilised product in dimethyl sulphoxide (DMSO) to a
concentration of 10 mM stock solution. The working solution was then prepared each time it was required, warmed to 37 °C and then used immediately to retain the fluorescence.

RCE cells were passaged and the cell suspension was then centrifuged. The supernatant was aspirated to remove the trypsin and culture medium taking care to leave the RCE cell pellet undisturbed at the bottom of the test tube. CellTracker™ green working solution was made up at a concentration of 15 µM in DMEM: F12 basal medium without foetal bovine serum (FBS) or any other supplements and warmed to 37 °C. The RCE cell pellet was then resuspended in the CellTracker™ working solution and RCE cells were incubated for 1 hour in suspension within the centrifuge tube wrapped in foil at 37 °C 5 % CO₂.

Following the incubation period, the RCE cell and CellTracker™ solution was centrifuged again and the CellTracker™ working solution was aspirated off leaving the stained RCE cell pellet. Using warmed complete RCE cell medium containing 1 % penicillin/streptomycin (Fisher Scientific, UK), the RCE cell pellet containing the stained cells was resuspended at the correct volume to obtain a cell concentration of 1 x 10⁶ RCE cells/ml. On the PDMS sample to be used in the device 295 µl of warmed complete RCE cell culture medium and 1 % penicillin/streptomycin were added to the PDMS well insert already positioned on top of the base PDMS as previously described in Section 6.2.3 of this Chapter. This contained the medium and RCE cells to a specific area of the PDMS sample. 5 µl of the CellTracker™ stained RCE cell suspension was added to the medium in the well to achieve a cell concentration of 5 x 10³ cells/cm² on each PDMS sample.

The plate was covered in foil to protect the fluorescent CellTracker™ dye from leaching and incubated for 24 hours at 37 °C 5 % CO₂ to allow the RCE cells to attach to the surface of the PDMS. As the device held two PDMS samples, for each run two PDMS samples were prepared and seeded for each experiment.

6.2.4. Assembly, imaging and stretching PDMS samples with the mechanical stimulation device

The assembly and disassembly of the mechanical stimulation device was carried out within a BSC as biological samples were used. To begin with, on a sterile 1 well plate turned upside down, a grid pattern was drawn using a fine permanent black marker pen on the underside of the plate of 0.5 cm divisions. The grid pattern was drawn on the central region of the plate so that when the PDMS – RCE cell sample was placed inside the 1 well plate in the centre, the grid pattern could be seen through the clear PDMS, as shown in Figure 61.
Figure 61 – A representative diagram of the grid pattern on the underside of the 1 well tissue culture plastic plate showing the PDMS sample and well insert, highlighting the area used for RCE cell imaging.

The 1 well plates containing the PDMS–RCE cell samples that had been stained using CellTracker™ Green CMFDA dye were placed in the BSC and each of the aluminium clamps supporting the samples during incubation were removed using sterile tweezers. The PDMS samples were then placed in the sterile, grid–marked 1 well plates flat on the base of the plate in the centre so that the grid pattern was visible through the plastic and PDMS and lined up with the well insert on the PDMS sample where the RCE cells were seeded. The area where the PDMS sample was placed in each plate was also marked so that it could be placed back in the same position following stretching. Tests were carried out prior to preliminary experiments that showed that the dimensions of the PDMS sample did not change following stretching. This was done by measuring the dimensions of the PDMS sample before and after stretch, which showed that reversible deformation was produced in the PDMS samples under the stain regimes described previously in Section 6.2.2.2. Table 8 below shows the measurements taken to test the sample deformation.
Table 8 – Showing the values for length of PDMS sample before and after stretch at 10%, 20% and 30% strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Before stretch 0 hrs average (cm)</th>
<th>After stretch 1 hrs average (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>20%</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>30%</td>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 8 showed that following stretching for the maximum time, the PDMS sample once released from the clamps had not deformed irreversibly under strain and its shape did not drastically change. This shows that the change in shape of the PDMS samples was not a major contributing factor to any difficulty experienced when aligning the PDMS sample on the grid marked 1 well plate for imaging before and after stretch.

The PDMS samples in their grid marked plates were then imaged using fluorescence microscopy (Nikon Eclipse TI inverted microscope, Nikon, UK) and images were focused within the 1 cm$^2$ well insert area where the RCE cells were seeded. In Figure 61 the PDMS sample was outlined in blue showing the arrangement of the sample in the plate and the grid pattern can be seen through it. The PDMS well insert was outlined in red and the well cut out in the insert was outlined in purple. In the 1 cm$^2$ area outlined in purple, the grid can be seen with four squares, which was used as a guide for imaging RCE cells cultured in this area on the PDMS sample. Within this area the central grid was imaged and RCE cells within the four squares of the quadrant were imaged before and after stretching using fluorescent and brightfield images. Images were taken using the Nikon Eclipse Ti microscope and digital camera.

Following fluorescent imaging before stretching, the PDMS–RCE cell samples were ready to be assembled into the device. The parts of the device were assembled and before the PDMS-RCE cell samples were assembled into the device, the clamps were removed from the PDMS samples and bulldog clips were used to attach the PDMS samples to the device, as shown in Figure 62. Using tweezers the PDMS samples were carefully moved from the grid-marked plate to be clipped on to the device on each end using bulldog clips, attaching to a stationary end and the moving rod end of the device.

The PDMS–RCE cell samples were suspended at this point due to the setup of the device, as shown in Figure 62. The PDMS-RCE cell sample was then straightened within the device by keeping the
sample under tension so that there was no buckling of the PDMS in the centre prior to the application of mechanical stimulation. The RCE cell culture medium was contained within the PDMS well insert on the PDMS sample.

**Figure 62 - Side view of the PDMS – RCE cell sample showing the PDMS well insert and the setup of the sample within the device.**

Once both samples were clipped in position, the plastic cover was placed over the device, covered in foil and transported to an incubator where the experiment would be carried out to control the temperature at 37 °C without humidity or CO₂. The different strain regimes were programmed onto the device prior to the assembly of the run using a computer and the Arduino software. Once the device was placed in the incubator, power was applied to the device and this started the static stretch programme being investigated.

After the stretch programme had completed for the duration of time being investigated, the device containing the PDMS–RCE cell samples was removed from the incubator and transported back to the BSC. The samples were taken out of the bulldog clips attaching them to the device and placed back into the grid marked 1 well plates. This was done ensuring the samples were placed in the correct plate and were lined up to all the position markings to ensure imaging after stretching was as accurate as possible. This was ensured by lining up the central well insert on the PDMS sample with
the four square grid in the centre of the plate markings as shown in Figure 61. This grid was marked out on the 1 well plate to the dimensions of the PDMS well insert area where RCE cells were seeded. Aligning each sample so that the central grid could be visualised through the centre of the well insert allowed an easier method of sample and plate alignment for imaging. Again images were taken in the central grid and RCE cells within the four quadrants were imaged after stretch. Representative images of before and after stretching as well as how the grid positions were used are shown in Figure 63.
Figure 63 – Images showing how the grid markings were used to image RCE cells before and after stretching. A1 – A6 show images for 30% strain at 0 and after 15 minutes of stretch, B1 – B6 show images for 20% strain at 0 and after 1 hour of stretch. Brightfield and fluorescent images showing CellTracker™ staining of live RCE cells taken at 10X magnification, scale bar shows 100µm scale. White arrows highlight the grid markings, showing the central grid marking and also the bottom left square of the central grid.
Figure 63 shows how the grid markings on the underside of the 1 well plate were used to align the images taken before and after stretch to the correct position to compare RCE cells within the same area. Images A1, A4, B1 and B4 show the grid markings in focus on the underneath of the 1 well plate. The rest of the images in Figure 63 show the RCE cells in the corresponding grid region in focus, in brightfield and FITC fluorescent channel. Figure 63 shows that this method of image alignment could be used to image the same region of interest on the PDMS-RCE cell sample before and after stretching. It proved to be a useful guide for identifying a particular area located in the central grid of the PDMS-RCE cell sample. However, it did not provide a way to track a specific individual RCE cell but more the RCE cells that were present in that region of interest.
6.3. Results

The device runs carried out contained two samples per run and the uniaxial stretch applied was 10%, 20% and 30% of the length of PDMS between the bulldog clips. Two sets of device experiments were performed at time points 0 and 15 minutes of stretch and also 0 and 60 minutes of stretch and both sets were carried out at 10%, 20% and 30% stretch. For each condition, brightfield and fluorescent images were taken of RCE cells stained with CellTracker™ green cultured on the surface of the PDMS samples prepared for the stretching device (Section 6.2.3) before (0) and after (15 and 60 minutes) stretch. The positioning of the uniaxial stretch meant that the PDMS–RCE cell sample was stretched through the central area for the particular duration of time and strain being investigated. Each of the time duration conditions were performed in separate experiments as it was found to be the easiest method to maintain sample integrity during assembly and disassembly of the PDMS-RCE cell sample for imaging.

The time points of 15 and 60 minutes stretch were chosen in order to see if there were any short term changes to the RCE cells following stretch. Due to live RCE cells being tested, the duration of stretch was chosen to maintain the integrity of the RCE cells and to minimise disruption to the samples during device runs. Static stretch was used to study the impact of mechanical stimulation on the PDMS–RCE cell samples. The percentage displacement used was 10, 20 and 30% of the length of PDMS between the bulldog clips to stretch the PDMS-RCE cell samples by the required strain (see Table 7) and these were chosen based on the capability of the device and the linear actuator movement. The programme controlling the movement of the rod was adjusted for each stretch condition and was easy to change when required. The device was not able to move the rod any less that these stretch conditions (Table 7) as the actuator did not have the ability to perform much finer movements.

6.3.1. Live RCE cell images of before and after stretch over different time points

Figures 64–67 show representative images from two experimental runs for each time point across three different strains used. Position 1 and position 2 represent the two sample positions used in each device run and the images shown are from each sample position for each stretch condition. The time points used were separated into 0 and 15 minutes (Figures 64 and 65) and 0 and 60 minutes (Figures 66 and 67) for each of the sample positions.
Figure 64 – Position 1 time points 0 (before stretch) and 15 minutes (after stretch for 15 minutes) over 3 different strains. Showing brightfield and FITC images for each condition, 10X magnification. Scale bar showing 100 µm scale.
Figure 65 – Position 2 time points 0 (before stretch) and 15 minutes (after stretch for 15 minutes) over 3 different strains. Showing brightfield and FITC images for each condition, 10X magnification. Scale bar showing 100 µm scale.
Figures 64 and 65 show representative images for time points 0 and 15 minutes after stretch at 10 %, 20 % and 30 % for position 1 (Figure 64) and position 2 (Figure 65). In the images for 10 % stretch in Figure 64, the highlighted area showed a shift in the images between time points 0 and after 15 minutes of stretch. This could have been due to the alignment method used but the same area of RCE cells could still be observed as highlighted. No significant change was observed in the RCE cells after 15 minutes stretch at 10 % within the highlighted area of the images, suggesting that this duration of stretch and the strain exerted did not affect the RCE cells on the surface of the PDMS. However other areas such as the detaching RCE cells that were visible before and after stretch (Figure 64, 10 %), did not appear to change or move position when investigating RCE cells within the highlighted area. These cells are smaller, more rounded cells that appear brighter in both brightfield and fluorescent images. Overall, there was no significant change in the RCE cell morphology, orientation or migration following 10 % stretch when comparing before stretch and after 15 minutes of stretch.

At 20 % stretch in Figure 64, the highlighted areas in the images showed that there was a large shift in the images when comparing the 0 time point and 15 minute time point. This would have been due to the alignment method and at 20 % stretch after 15 minutes the images showed greater chance of misalignment than 10 % stretch at the same time points. However the RCE cells highlighted were visualised before and after stretch but did not seem to change in their shape or orientation when before and after 15 minutes of stretch images were compared. After 30 % stretch for 15 minutes, the highlighted area of RCE cells was observed before and after stretch but there was a shift between these images again. No significant changes in the RCE cells was observed following 30 % stretch at 15 minutes and it is not clear from the images if any true cell translocation in response to stretch can be distinguished from the misalignment of the PDMS-RCE cell sample in the before and after stretch images. Also it was observed in the brightfield image after stretch at 30 % that the RCE cells appeared more rounded in their morphology and therefore seemed as though they may have been detaching from the PDMS following stretching. However, the FITC fluorescent image for 30 % stretch after 15 minutes showed that the morphology of the RCE cells before and after stretch appeared to be similar and there was no significant change in the shape and orientation of the RCE cells (Figure 64).

Figure 65 also showed representative images for 10 %, 20 % and 30 % stretch for time points 0 and 15 minutes after stretch but were for position 2, the second sample position in the device. This also showed that over the three different stretch conditions, after 15 minutes there was no significant change in the morphology, orientation and migration of the RCE cells. For 10 % stretch in Figure 65,
there was a greater shift in the images when comparing before and after 15 minutes of stretch, suggesting that the grid marking method had not quite worked for this sample. However the RCE cell morphology after 10 % stretch was not dissimilar to the cell morphology before stretch as was the case with images for 20 % stretch in Figure 65. Some of the RCE cells in the images for after 10 %, 20 % and 30 % stretch were observed to be more rounded, which suggested that these RCE cells were detaching from the surface of the PDMS. One explanation for the change in appearance of the RCE cells after 30 % stretch is that the stress on the RCE cells was too great and this may have caused the cells to begin to detach from the PDMS surface, disturbing the attachment. Therefore this could have impacted on the image alignment if RCE cells had detached and moved. The image in Figure 65 after 30 % stretch is not clear enough which could have been due to misalignment. The overall morphology and orientation of the RCE cells in Figures 64 and 65 showed that after 15 minutes of static stretch at 10 %, 20 % and 30 %, no significant changes were observed in the RCE cell shape or migration. However, it was apparent that there was an issue with misalignment due to the shift in before and after stretch images and therefore any migration of RCE cells would have to be studied further to distinguish this from any other effects of stretching on the sample and alignment.

It was thought that the stretching of the RCE cells and the shift in the images before and after stretch following all the strain regimes could have been as a result of hysteresis of the PDMS elastomer. Hysteresis of an elastomer occurs between the loading and unloading of the elastomer as there is a lag in the length of the unloaded elastomer compared to the loaded elastomer against the force applied (Deguchi et al., 2015). The unloading curve appears to show less stress that the loading curve in the same cycle and more energy would be required during loading compared to unloading the elastomer, meaning that the area in the hysteresis loop between the curves represented the dissipated energy (Lee et al., 2016). This typical viscoelastic response to loading could have explained the difficulty in alignment of the PDMS-RCE cell samples after stretch. The PDMS-RCE cell sample was removed from the device and bulldog clips and was then transferred to the 1 well grid marked plate. This process added a short delay of approximately 10 minutes before samples could be imaged. However, PDMS as an elastomer can be deformed reversibly under small loads due to its elastic properties (Hopf et al., 2016). At higher strain, a viscoelastic material may exhibit a time-dependent increase in strain, which is known as viscoelastic creep (Beer, 2012). This may relate to the static mechanical behaviour observed in the PDMS samples following stretching and could have contributed to the positioning of the PDMS-RCE cell sample before and after stretching, increasing the chances of misalignment of the PDMS-RCE cell samples to the grid marking on the 1 well plate when imaging.
However, the duration of the experiments shown in Figures 64 and 65 was 15 minutes of stretch at 10 %, 20 % and 30 % stretch, was a shorter length of time for stretching the PDMS. This may not have been a long enough duration under load for the PDMS to display viscoelastic creep properties (Wen and Janmey, 2013). Also the PDMS – RCE cell sample was aligned on the grid marked 1 well plate outside of the device in the same way for before and after stretch images and it was also ensured that there was no significant change in the size of the PDMS after stretching as described previously in Section 6.2.4. The material response to stretching and unloading may have had an impact on the position of the RCE cells in the area of interest due to alignment of the sample but as shown in Figures 64 - 67, the same or similar RCE cell areas were found after imaging in most conditions. However potential structural changes to the PDMS polymer and therefore changes to the mechanical properties of the PDMS following stretching were not measured so it could not be ruled out that changes in the PDMS elastomer had occurred after stretch.
Figure 66 – Position 1 time point 0 (before stretch) and 60 minutes (after stretch for 60 minutes) over 3 different strains. Showing brightfield and FITC images for each condition, 10X magnification. Scale bar showing 100 µm scale.
Figure 67 – Position 2 time points 0 (before stretch) and 60 minutes (after stretch for 60 minutes) over 3 different strains. Showing brightfield and FITC images for each condition, 10X magnification. Scale bar showing 100 µm scale.
Figures 66 and 67 show representative images for time points 0 and 60 minutes after stretch at 10 %, 20 % and 30 %. After 10 % stretch (Figure 66), a shift in the images at time point 0 and 60 minutes can be seen, which suggested that the grid marking method for aligning PDMS–RCE cell samples before and after stretch did not always work as effectively. However the highlighted area of the images showed that after 10 % stretch for 60 minutes, there was no significant change in the morphology, orientation or migration of the RCE cells (Figure 66).

For 20 % stretch after 60 minutes (Figure 66), there was also a shift when before and after stretch images were compared however the same area of RCE cells could be seen in both images. The RCE cell morphology had not changed significantly following 20 % stretch for 60 minutes and no major differences could be observed between the 0 and 60 minute time point images (Figure 66). Both brightfield images of RCE cells before and after 20 % stretch for 60 minutes were observed to be slightly raised in appearance. This could have been due to the cell culture medium covering the RCE cells and the refraction of the light path through the PDMS sample and culture medium, giving the RCE cells a more rounded appearance. Slight variations in the thickness of the PDMS could also have contributed to a small change in appearance of the RCE cells. As both before and after stretch images appeared this way, it was not thought to be a result of stretching but more to do with the PDMS sample itself (Figure 66).

It was more difficult to align the before and after stretch images following 30 % stretch after 60 minutes (Figure 66). Similarly, to 10 % and 20 % strain after 60 minutes stretch, no significant differences were observed after stretch. As observed for 15 minutes of stretch (Figures 64 and 65), the misalignment of the PDMS-RCE cell samples meant that it was not clear if there were changes in cell migration. This may have been related to the deformation of the PDMS-RCE samples at the higher strain for a longer period of time compared to 15 minute stretch times (Figures 64 and 65).

In Figure 67, representative images of 10 %, 20 % and 30 % stretch are shown also for before and 60 minutes after stretch but are from the second sample position in the device, position 2. The images obtained from these samples were better when compared to the position 1 images in Figure 66 and the PDMS–RCE cell samples were more successful in terms of cell density in each area and the alignment of images before and after stretch.

After 10 % stretch for 60 minutes, the RCE cell morphology was very similar between the before and after images. The alignment of the before and after stretch images using the grid marking method had worked well and the same area of RCE cells could be observed before and after stretch (Figure 67). It was also observed that there were a few more detaching RCE cells in the images after 10 %
stretch for 60 minutes, as seen by their smaller, rounder and brighter appearance in both the brightfield and fluorescent images (Figure 67). This could have been due to the condition of the RCE cells during the experiment, change in the environment or as an effect of the manipulation required of the sample for imaging. Or this may have been related to the stretching of the sample and the experimental conditions. However, there were no significant changes to RCE cell morphology or migration after 10% stretch for 60 minutes.

The representative images shown for before and after 20% and 30% stretch (Figure 67) were aligned quite well and the highlighted area of interest could be seen clearly in the before and after stretch images for 20% stretch. There was more of a shift in the before and after stretch images for 30% stretch (Figure 67) and as observed in Figure 66, there were some misalignment issues. There were no significant differences in RCE cell morphology, orientation or migration between the before and after stretch images for 20% and 30% stretch after 60 minutes. Some of the RCE cells appeared more rounded, which could suggest that they were beginning to detach from the PDMS surface due to the strain on the PDMS substrate or because of the longer period of time within the device.

For 0 and 15 minutes and 0 and 60 minutes at 30% stretch, alignment of the RCE cells using the grid marking method proved to be more challenging compared to the other stretch percentages after 60 minutes stretch. Further investigations into the bulk mechanical properties of the PDMS after stretching and analysis of RCE cell viability and function before and after stretch would be required in order to understand the processes involved within the cell following mechanical stimulation of modified substrates. The highest strain on the PDMS samples caused problems with sample alignment for imaging and may have been due to mechanical changes occurring within the PDMS as the stretch was applied over time.

Hysteresis and strain rate dependence were found to be less prominent in the PDMS elastomer when compared to other conventional elastomers (Hopf et al., 2016; Lee et al., 2016). However, this would require further investigation to measure the effect of time between stretch and imaging on the recovery of the PDMS sample shape and also how the stretching of the PDMS samples affected the bulk mechanical properties.
6.4. Discussion

6.4.1. Overall findings from the preliminary stretching experiments of RCE cells

When the findings from the PDMS-RCE cell sample stretching experiments were compared, the outcomes were summarised into a few key observations from the experimental conditions used. It was found that from observing the images collected in Figures 64-67, there was no significant change in cell morphology, orientation or migration across all conditions and time points investigated for static stretching. There were slightly more detaching or already detached RCE cells observed in the 60 minute stretch condition compared to only 15 minutes of stretch. This could have been due to the condition of the RCE cells during the experiment, the longer time that the PDMS–RCE cell samples were kept in the device or the additional handling of the sample that was required when imaging RCE cells before and after stretch. All of these factors combined may have impacted on the RCE cells and further investigation is required into the effects of the stretching device on the viability of the RCE cells in terms of the duration and amount of stretch exerted on the RCE cells and the different stages of device assembly and disassembly. The viability of the RCE cells was measured before each PDMS sample was seeded for the device and RCE cells used were over 90 % viable for each experimental run at the sample seeding stage. Therefore, at various time points during the sample loading, running of the device and imaging, more information on the RCE cell viability is needed to further understand the impact of these conditions on the RCE cells.

A major outcome of the preliminary stretching experiments is correlating the location of the PDMS-RCE cell samples for imaging before and after stretching. This is crucial for sample alignment and accurate tracking of RCE cells as the PDMS substrate is deformed during stretching. The challenge arises due to the elastic behaviour of PDMS and how it responds to deformation, changing dimensions under higher strain for a longer duration where other mechanical characteristics of elastic and viscoelastic materials may have a role. By improving image correlation, this will enable the determination of RCE cell changes that occur due to mechanical stimulation, for example, changes in RCE cell orientation and migratory behaviour, and not due to the misalignment of the PDMS-RCE cell sample when imaging. This has been discussed further in Section 6.4.2 where the device design has been critically reviewed.

6.4.1.1. Mechanical stimulation effects on the cytoskeleton and cell alignment

The cytoskeleton of the cell is the basis of cell movement, comprising of actin microfilaments, microtubules and intermediate filaments, that are cell type specific (Holle et al., 2017). The actin microfilaments are known to have a crucial part in stretch – induced cell alignment, so much so that
if the actin cytoskeleton is disrupted the cell alignment in response to stretch will not occur (Kaunas, Usami and Chien, 2006; Goldyn et al., 2009). The alignment and orientation of cells parallel to uniaxial stretch has been discussed in the literature and previous studies on cell alignment of NIH 3T3 fibroblasts following a static uniaxial tension showed that there was initial cell alignment and elongation parallel to the applied stress, which became more pronounced the longer the stimulation (Steward et al., 2010). Pang et al (2011) also showed that after static uniaxial stretch initial cellular alignment was observed parallel to the strain axis. However they used collagen hydrogels as substrates and found that collagen alignment occurred as a result of the observed cell migration along the direction of stretch (Pang et al., 2011). An important point is that cells respond to the applied stretch in different ways and there are several factors that can affect this. The type of cell and its contractile state, the type of strain applied, and the mechanical properties of the substrate used for cell culture can all impact on the cellular response to stress (Sears and Kaunas, 2016).

In the literature the duration of static stretch was reported as much longer than the experimental conditions shown in Figures 64–67, with research exploring 12 hour and 24 hour cultures and others investigating responses to mechanical stimulation after several hours (Gupta and Grande-Allen, 2006; Steward et al., 2010; Pang et al., 2011; Li et al., 2014). A longer duration of stretch would have allowed a greater response to be observed in the RCE cells. The stretching device results show in Figures 64–67 investigated short term stretch at 15 minutes and 1 hour and this may not have been enough time to observe significant differences in cell alignment, orientation or morphology. However the current design of the stretching device would not have been able to support the RCE cells for long term experiments and would have impacted on the cell viability and response to stretching. Further considerations would have to be made in order to modify the device to be able to consistently perform longer experiments whilst maintaining the integrity of the PDMS-RCE cell sample. Also improvements are needed for the image correlation, which has been discussed further in Section 6.4.2.

Further work into the effects of the stretching experiments on focal adhesions and their presence and type would be interesting to improve understanding of the response of the RCE cells to stretch. It is known that living cells do not adhere to the PDMS surface without pre-treatment and in the literature it is common to use ECM coatings to promote cell attachment (Colombo et al., 2010; Palchesko et al., 2012; Chang et al., 2013; Schurmann et al., 2016). During the stretching experiments discussed in this work, PDMS-RCE cell samples were not coated in an ECM such as collagen or fibronectin for example but the surface of the PDMS was treated with oxygen plasma before the RCE cells were seeded on the surface. It was suggested that the RCE cells were laying
down their own ECM as they formed attachments to each other and the PDMS surface. Actin microfilaments form contractile bundles or stress fibres, which are important for certain cell functions such as cell alignment, contractility and adhesion and are strain–bearing structures. These stress fibres anchor to focal adhesions such as integrins and other proteins. The focal adhesions link the actin cytoskeleton to the ECM and it is through this connection that the actin cytoskeleton and therefore the cell senses mechanical forces (Sears and Kaunas, 2016).

The alignment of cells stimulated by stretching has been shown to be reliant on the actin cytoskeleton of the cell, while the intermediate filaments were important for the mechanical integrity and organisation of cells but had a relatively small part in stretch–induced morphological changes (Anselme, Ploux and Ponche, 2010; Sears and Kaunas, 2016). These cellular structures facilitated the elongation of the cells parallel to the stretch and responded to the mechanical stimulation through focal adhesions, linking the substrate surface to the cell through integrin mediated cell–matrix adhesion. This was also the mechanism of mechano-sensing of the cell in its environment and the actin cytoskeleton would generate mechanical forces by contracting in response to a substrate or during cell spreading (Anselme, Ploux and Ponche, 2010). Further work would be required to understand what is happening within the cell on a molecular level following stretching and what a particular level of strain exerted on the cell would affect in terms of cell behaviour and function.

Imaging of focal adhesions and cytoskeletal structural changes, in particular the actin cytoskeleton and vinculin, a universal marker for focal adhesions in cells, using immunostaining techniques would be useful to visualise any changes before, during and after stretch of the RCE cells cultured on PDMS to further understand the alignment and orientation changes in the RCE cells that were beginning to be observed following the preliminary results shown in Figures 64-67. The CellTracker™ stain used in experiments on the live RCE cells stained the whole cell and did not distinguish between important features. However in order to obtain clear well defined images using immunostaining, the samples would have to be fixed whereas the results shown in Figures 64-67 could have shown changes that occurred in living RCE cells as the RCE cells seeded onto the PDMS surface were live cells and were not fixed at any point during this process. Also if any markers for key cellular processes such as cell migration or proliferation are up - or down – regulated as a result of this stimulation would be important to study further. For example, cell adhesion molecules are instrumental in cell migration and integrin and cadherin adhesion systems could contribute to mechanical signalling and coordinated migration of cells (Janmey and McCulloch, 2007).
The data obtained from the preliminary device runs did not clearly demonstrate that any changes observed in the morphology or location of the RCE cells after stretching were directly related to the amount of stretch exerted on the PDMS–RCE cell samples. There was not a clear distinction between the shift in the sample due to misalignment and potential RCE cell translocation or any other responses to stretching and therefore the data did not show any significant change in RCE cell morphology or behaviour following stretching. It was uncertain if any movement of the RCE cells would be to do with the stretched PDMS being pulled in a uniaxial direction and therefore the RCE cells also move in that direction and align with the stretch as they are attached to the PDMS.

The elastic behaviour of PDMS meant that following static stretch, the PDMS would return back to its original shape as the elastomer can recover following deformation. If the material did deform plastically and therefore did not return to its original shape after the deforming force or stretch was removed, the resulting PDMS sample shape may then have impacted on the position of the RCE cells on the surface as it had deformed. Elastic creep of a material is the delay in the material returning to its original shape after the deforming force or stretch has been removed. This could also have had an impact on the position of the RCE cells following stretch as explained previously however the after stretch images were not taken immediately after performing the static stretch so again it was unclear whether this had affected the PDMS samples. An increased movement would be expected in the detached RCE cells as they were not attached to the surface of the PDMS sample and were able to move freely and this resulted in a more obvious change for these cells, which was expected.

Overall, as there were no dramatic changes in the attached RCE cells following stretching, the results could not confirm any significant behavioural or morphological changes in the RCE cells as a response to mechanical stimulation. Further investigation would be needed to obtain quantitative data in addition to the morphological and physical data from RCE cell imaging correlating before and after stretch images to accurately track RCE cells during mechanical stimulation. This would allow further conclusions to be made about the effects of mechanical stimulation on RCE cells in terms of their biology and function and how this impacts on key processes such as cell migration and proliferation.
6.4.2. Critical review of the current device design

Following the preliminary experimental runs of the stretching device, several issues and setbacks of the device design became apparent and these have been discussed further.

6.4.2.1. Assembly of the device

The device assembly prior to the PDMS–RCE cell sample assembly was fairly straightforward as the design of the device was very simple. There were two main arms of the device that extended over the linear actuator and either side of the actuator were the two positions for samples to be held. One arm had on each end one clamp for sample 1 and sample 2 and this was fixed onto the device. The other arm had on each end one clamp that fitted to the moveable end of sample 1 and 2. This arm was attached to the movable rod controlled by the actuator. This moved forward and by doing so stretched the PDMS sample (Figures 56, 57 and 58).

The samples were clamped into position using bulldog clips as these were simple and inexpensive to use and fitted the size of the samples. The other end of the bulldog clip was then hooked onto the corresponding arm of the device (Figure 56 and 57). This method of clamping the samples to the device was used because the original clamps did not work as effectively. The device arms and original clamps were made of aluminium as this was easily available and inexpensive to use. However this meant that heat sterilisation had expanded the metal and as it cooled altered its shape slightly and therefore the device parts especially the original clamps did not fit as well. If the device parts were to be made from metal for the improved device, stainless steel would be more appropriate as it would withstand high temperatures without warping and would be non-corrosive.

The bulldog clips worked well as a replacement for the original clamps for the preliminary experiments. Handling of the PDMS–RCE cell samples was done within a BSC, which was quite challenging to do when the samples were loaded in the device. This was due to the device being bulky and heavy as a thick plastic base plate had been attached underneath all of the device components. Although the base plate made it easier to carry the components of the device in one go, it was not compact or lightweight and therefore was more of a hindrance.

Due to the simple build of the device, a controlled environment was not incorporated into the device itself and during stretching the whole device was kept within an incubator at 37 °C, set to no humidity, so without water present in the incubator and no CO₂ gassing. The build of the device meant that the electronic components were fixed onto the end of the actuator and were not removable. In order to protect these parts no humidity was used whilst it was within the incubator during stretching. The incubator was not connected to CO₂ and the device was only exposed to atmospheric CO₂ and humidity. This incubator had to be used for device runs because it was not
Currently in use so there was plenty of space available for the device. For longer periods of time within an incubator with lower or no humidity, water from the cell culture medium will evaporate faster and this could have a negative impact on the osmotic balance in the cells. However in this case the maximum duration that the PDMS-RCE cell samples were left within the incubator without humidity was 1 hour and the level of cell culture medium was checked within this time. A small volume of 300 µl medium was used on each PDMS-RCE cell sample.

Regarding no CO₂, the cell culture medium used contained HEPEs buffer and this maintains the pH to within the target range and around pH 7.4. A constant supply of CO₂ maintains the equilibrium between the water and buffer within the culture medium but the buffer can slow the rate of pH change (MacKenzie, MacKenzie and Beck, 1961; Taylor, 1962). Therefore for a short period of time, it was thought that these conditions would be suitable for the PDMS-RCE cell samples. Longer experiments would need a more controlled environment for the cells with continuous monitoring to ensure the stability of the environment and cell viability measurements during longer experiments would be required.

6.4.2.2. Assembly and disassembly of the PDMS – RCE cell samples within the device

Due to the PDMS–RCE cell samples being cultured separately in individual 1 well plates and not in the device itself, there was an additional manipulation or handling of the sample required and this may have disturbed samples as they were being loaded into the device or removed from the device for imaging. The PDMS–RCE cell samples had to be handled with tweezers and were always handled within a BSC, which also added to the manipulation of samples. The bulldog clips that were used as clamps worked well but it was difficult to assemble the samples in them in one go so this added to the handling of the sample. However they were easier to disassemble when compared to the original clamps as these had screw fastenings.

Ideally the device would be compact enough to hold the PDMS samples within it whilst the samples were seeded with RCE cells, cultured for 24 hours and then stretched so that the sample would not need to be disturbed by removing it out of the culture plate in to the device. However if this was the case, the device would also have had to be a device that would allow the cells to be imaged in situ with the samples loaded within the device and be compact enough to be kept on the platform of a microscope. This would allow the same area of the sample to be imaged either at certain intervals during stretch (real time imaging) or after stretching but without the disassembly of the sample from the device to image the cells.

In order for the device to function as an online imaging device it would have had to be compact, portable and lightweight enough to be kept on the microscope stage throughout the experiment.
without disrupting the function of the microscope. The device would have been enclosed but an environment of 5 % CO₂ would be required within the device to control cell culture conditions during the experiment for an online device. This would also depend on the buffer system used within the cell culture medium but the buffer will only slow the rate of pH change so this would need to be considered for long term experiments. Also the base of the sample holder and device had to be clear and thin enough to image the cells through the base of the device as well as the PDMS sample as the microscope used has limited length of focus relating to the working distance of the 10X objective used in preliminary experiments. A heating element would be required within or around the device to maintain the temperature within the device at 37 °C to ensure that the cells were in the optimum environment but ensuring that the base of the device was left clear with no obstruction to imaging.

Maintaining these conditions within an online device could be challenging and therefore at this stage of the project the device remained offline because this meant that the whole device could be kept within an incubator during the experiment. The current device could have been improved by producing a more compact and portable version that allowed the PDMS–RCE cell samples to remain within the device once loaded and that could be imaged within the device without any need to remove samples or disrupt them in any way. However, this would have required a way to detach the sample holder section of the device from the current mechanical parts as they would not have been able to fit onto the microscope stage together.

A redesign would require a more compact actuator to be sourced or alternative mechanical parts that would fit the requirements in order to deliver an improved device. The current device had a PDMS well insert that was placed on top of the PDMS sample which allowed the cells and culture medium to be contained within the well. This meant that only small volumes were required and a whole chamber or the whole device did not need filling with cell culture medium. The assembly and disassembly of the samples in the device were more straightforward as large volumes of cell culture medium did not need to be handled. The well insert is discussed in more detail in the next section.

6.4.2.3. PDMS Well insert and cell seeding area
The well insert was useful for the purpose of containing the RCE cells to the central area of the PDMS and therefore there was no need to completely cover the whole PDMS sample with cell culture medium. The device was not sealed and therefore the entire chamber would have to be filled with medium and this would not be feasible with the scale of the current device. Also the PDMS-RCE cell samples were not in their own containment within the device so having a well insert was the best approach for preliminary experiments.
However it was not further investigated whether adding the well insert to the surface of the PDMS sample would have an effect on the stretching of the PDMS sample and whether the effects of this would impact on the distribution of stress across the whole sample particularly the area where the cells were. The well insert was made from the same material and also was only 2 g of the uncured PDMS for the whole section but this was cut to a smaller size after curing. The approximate thickness of the PDMS well insert was 0.5 mm and in the literature it was found that a thickness of PDMS lower than 200 µm or 0.2 mm showed the point at which PDMS mechanical properties were dependent on sample thickness (Liu et al., 2009; Hopf et al., 2016) and the PDMS samples and well inserts used were well above this thickness level for thickness-dependent mechanical properties to be observed (see Chapter 2, Section 2.1.3 for sample thickness range).

Although the impact of the PDMS well insert on the mechanical properties of the PDMS sample as a whole was not further investigated, the well insert worked effectively to create an area on the PDMS sample to contain the RCE cells and cell culture medium. PDMS can bond to itself very well producing a watertight seal (Liu, Sun and Chen, 2009). It successfully allowed the RCE cells to attach to the PDMS in a defined area and prevented leakages of cell culture medium before, during and after stretching. Once positioned on the PDMS sample in the correct location, the well insert did not need any further manipulation or modifications following cell seeding due to the binding qualities of PDMS to PDMS. There was also no need for an additional adhesive to attach the PDMS well insert to the PDMS base sample.

6.4.2.4. Tracking of cells before and after stretch using grid markings

The assembly and disassembly of the PDMS–RCE cell sample in the device was an issue as samples had to be disturbed for imaging because the current device was an offline device and real time imaging could not be incorporated. As discussed previously, the device was not compact and portable and a sample could not be imaged within the device but instead had to be transferred to a 1 well plate for imaging. This meant there was a need for a marking system or method of tracking the RCE cell area that was of interest or being imaged. This was a challenge as the marking method should not disturb the sample in any way that could interfere with the results following the stretching experiment. A simpler solution was used for the preliminary experiments and an external grid marking on the underside of a 1 well plate was used as described previously in the materials and methods section of this Chapter (Section 6.2.4). As the 1 well plate and PDMS sample were clear the grid markings could be seen through the sample and RCE cells on the surface of the PDMS sample could also be focused on using an inverted microscope.
As described previously in Section 6.2.4, a grid in the central area of the sample was used to image RCE cells attached to the surface of the PDMS sample and image RCE cells within this area of the grid markings. The grid markings were used as more of a guide to image RCE cells in a particular area of the PDMS sample. Using beads or markers within the PDMS sample would also have worked in a similar way as the PDMS–RCE cell samples would still have to be removed from the current device for imaging before and after stretching. However, the beads or markers within the PDMS would be used for image correlation when processing the images before and after stretch. This would aid RCE cell tracking and by correlating a bead or marker in a particular part of the PDMS sample over time, image processing software could be used to track RCE cell migration or other changes in response to strain and would enable the study of individual cell responses. Tracking beads could also help with confirming whether creep is observed in the PDMS material during stretching by using the positioning of the beads or markers before and after stretch (Style et al., 2014).

If a grid was etched into the PDMS itself this could impact on the mechanical properties of the PDMS and could cause changes in the structure of the PDMS due to the stretch. However, as the PDMS–RCE cell samples were kept within an incubator for cell culture at 37 °C there was some concern over whether the PDMS would be affected by heat expansion and whether this would impact on a grid etched into the PDMS or beads/markers within the PDMS.

The PDMS polymer can be heat cured and once the two parts of the Sylgard® 184 kit (base elastomer or pre-polymer and curing agent or cross-linker) are mixed at the correct mixing ratio (10:1 of base elastomer to curing agent), by heating to elevated temperatures the liquid mix turns into a solid cross-linked elastomer in a few hours (Dow Corning, 2014a). In the literature it had been found that by changing the ratio of base elastomer to curing agent, the mechanical properties of the cured PDMS can also change (Mata, Fleischman and Roy, 2005; Liu, Sun and Chen, 2009; Palchesko et al., 2012). It was also found that using very high curing temperatures can affect the mechanical properties of PDMS. Liu et al (2009) found that the mechanical properties of PDMS are related to its thermal stability and they found that if a curing temperature higher than 200 °C was used, it would cause thermal decomposition of the PDMS and therefore reduce the mechanical strength (Liu, Sun and Chen, 2009). This was supported by Johnston et al (2014), who also investigated the effects of heat curing on PDMS bulk mechanical properties ranging from 25 to 200 °C, which was the upper limit of the manufacturers recommended working temperature range (Johnston et al., 2014). Therefore the temperature of 37 °C was unlikely to have a major impact on the mechanical properties of the PDMS or the stability of the elastomer during experiments.
The mechanical properties and the way in which the uniaxial stretch was transferred through the material could have been affected by etching the surface of the PDMS sample. The material may not have been uniform in thickness, surface roughness and surface properties following the etching of a grid pattern into the PDMS, even though the etching may only be nanometres in depth. Further investigations were needed into this option for tracking the RCE cells before and after stretch to gain further insight into whether this type of grid marking would affect the PDMS sample properties, the RCE cells cultured on the sample or both.

The PDMS sample itself was not marked in preliminary experiments because of the deformation of PDMS during stretch. Although the elastic properties of PDMS allow it to deform reversibly under small strains, it was unsure as to whether the markings etched onto the PDMS would be affected by the deformation and therefore might change configuration due to the changes within the PDMS mechanical properties. Markings etched onto the PDMS sample were not tested but it was thought that at higher strain the PDMS could potentially deform irreversibly and this could impact on the tracking method employed. This is why the method of marking the 1 well plate was used to see if this could be used as a method for alignment as these markings would not change position. The PDMS sample could then be aligned to the grid markings before and after stretch to be able to image the same area of RCE cells after removing the sample from the device. With the sample alignment, additional markings were made on the 1 well plate to outline the placement of the PDMS-RCE cell sample before stretch and these markings were adhered to for alignment of the sample after stretch too.

However as discussed previously, this method of sample alignment before and after stretch was not accurate and human error in manually aligning the samples would have reduced the accuracy. It was difficult to distinguish any migratory response of the RCE cells from the misalignment of the PDMS sample in the image after stretch.

6.4.2.5. Stretching of PDMS–RCE cell samples

The current stretching device was only able to perform static stretch at one speed but the amount of stretch exerted on the PDMS sample could be changed. The forward movement of the rod attached to the linear actuator controlled the stretch of the PDMS and the particular amount of stretch was held for a certain amount of time by changing the programme as described previously in Section 6.2.2 of this Chapter. It was not possible to carry out cyclic stretching on the samples due to the limitations in the control capabilities of the actuator that was used. In the literature it was found that cyclic stretching of cells cultured on elastomeric substrates caused the actin fibres to align perpendicularly to the direction of the stretch and that without strain, cell migration was random.
and with no preferred direction (Moretti et al., 2004; Holle et al., 2017). Irrespective of cell type, cyclic uniaxial stretch, as opposed to static uniaxial stretch, was found to cause the cells to align perpendicularly to the direction of cyclic stretch and therefore the direction of minimal substrate deformation or stretch avoidance. The stress fibres, constituting the main cytoskeletal structures, respond almost immediately to stretch and align along the direction of minimal deformation, hence why the cells also align this way after time (Li et al., 2014). In the results obtained for the RCE cell–PDMS stretching experiments (Figures 64–67), static uniaxial stretch was used as opposed to a cyclic uniaxial stretch and Figures 64-67 show the direction of stretch applied. In some elastomeric materials, cyclic loading results in the Mullins effect and the typical viscoelastic response of hysteresis (Lee et al., 2016). The Mullins effect explains how rubber–like materials will show a considerable change in their mechanical properties as a result of the first extension or loading (Diani, Fayolle and Gilormini, 2009). However, Mullins effect, hysteresis and strain rate dependence was found to be less prominent in the PDMS elastomer (Meunier et al., 2008). The ability to perform cyclic stretching would be a useful improvement to the device and it would be interesting to see what effect cyclic stretching had on the RCE cells in terms of cell alignment and migration.

Also a more precise and more sophisticated system for controlling the stretch is desirable whereby the accuracy of the current device will be improved. This would also provide greater control over the position of the moveable rod and better programming of the actuator, providing more control over the amount of stretch exerted on the PDMS sample. The system would allow finer movements of the rod due to better control over the linear actuator and by being able to move the rod smaller distances, more accurate and specific changes in the stretch parameters can be made for the PDMS–RCE cell samples.

More options for adjusting the frequency and speed of stretching in particular for cyclic stretch could be interesting to see the effects these changes have on RCE cells attached to PDMS substrates. However this would have to be incorporated into the device using a different linear actuator and servo or other mechanical parts that had the capability to adjust these parameters, giving the user more control over the experiments. More mechanical testing is needed to understand what effects are exerted on the PDMS sample following stretching and what impact cyclic stretching has on the mechanical properties of PDMS and the changes if any that occur in the RCE cells before and after stretch.

The clamps used during stretching experiments were bulldog clips, which worked effectively as a replacement for the original clamping mechanism (described in more detail in Section 6.4.2.1). However because the PDMS samples were clipped into them, the stretch may have been distributed
unevenly across each edge of the PDMS sample and this could have impacted on the stretch exerted across the whole PDMS sample. Extra care had to be taken when positioning the sample in the bulldog clip on each end and it was difficult to do this accurately in one go using tweezers so there would inevitably have been more sample handling. Overall, using bulldog clips worked effectively as a replacement for the clamps designed for the device and they were readily available and cost effective. However as they were not custom made for the device, the fit was not as accurate as the original custom made clamps but they were able to be used in the stretching device and did not appear to have any adverse effects on the stretching mechanism.

6.4.2.6. Imaging the PDMS – RCE cell samples before and after stretch

For imaging before and after stretch, the PDMS samples had to be placed in the grid marked 1 well plate (see Section 6.4.2.4) for imaging as images could not be taken of the RCE cells whilst the PDMS samples were in the device. This meant that there was an inconvenience of transferring samples to a different plate for imaging before stretch and also for unloading the sample from the device for imaging after stretch. However this made the device build simpler because if the samples were able to remain within the device during imaging, as explained above in Section 6.4.2.2, it would need to be compact, portable, have gas and temperature control and be made out of a clear, lightweight plastic or other material through which the sample could be imaged.

The alignment of images taken before and after stretch of the same RCE cell area was the main issue with imaging, as described in Section 6.4.2.4 and the method used was the best solution with the resources available. The grid marking method could be improved further or an alternative method to improve imaging of the same RCE cells before and after stretch could be investigated further. It was observed that fluorescent images taken of the RCE cells stained with CellTracker™ green tended to decrease in their fluorescent signal after stretch when compared to the before stretch images. This was seen across all conditions and was expected due to the handling of samples and the time taken to assemble and disassemble the device and remove samples from the device. This may have caused the RCE cells to lose some of the fluorescent dye intensity and as they were live cells and metabolically active, this process may have been accelerated compared to fixed cells that have been stained. Increased handling of the PDMS–RCE cell samples may not have been the most ideal method for preserving the fluorescent dye in the RCE cells but it was the easiest way to image the samples with the current device design. However for the duration of time that the PDMS–RCE cell samples were used during the preliminary device runs, it was found that the reduction in the fluorescent signal in samples did not greatly affect the imaging of the RCE cells after stretch and clear images could still be taken of the cells.
Higher magnification images would allow more detailed analysis of changes to RCE cell morphology following stretching but due to the working distances of the microscope objectives, increasing the magnification above 10X would reduce the working distance. Therefore the PDMS sample and grid marked 1 well plate would have to be thinner in order to be able to image the RCE cells on the surface of the PDMS sample at higher magnifications. A lower magnification such as 4X could also be used to get an overall view of the PDMS-RCE cell sample area and the global response of RCE cells to stretching and general trend in behaviours could be observed. Using image analysis software such as ImageJ could be incorporated into further experiments in order to measure and quantify RCE cell changes as a response to stretching such as the orientation, migration and morphological changes of the RCE cell. If imaging at higher magnifications could be used going forward, more detailed analysis could be performed on individual RCE cells using ImageJ or another image analysis software after stretching.

6.4.2.7. Disassembly of the device after stretching experiments

Device disassembly was fairly straightforward but after stretch the whole device, which was quite bulky due to the base plate, had to be removed from the incubator used for experiments and transferred to a BSC in order to remove the PDMS samples from the device. At this stage removing the PDMS samples from the bulldog clips was straightforward as the samples were to be discarded. The rest of the device was easy to disassemble but was time consuming to take apart and clean. The grid marked 1 well plates used for imaging of PDMS–RCE cell samples were also disposed of as they were not reusable.

Some parts of the device were not suitable for heat sterilisation and were cleaned using 70 % IMS. Ideally if all parts of the device could be heat sterilised this would have made cleaning the device parts more straightforward and ensured sterilisation for the next experimental run. However there were several elements of the device to consider when materials were chosen, as well as the resources that were available, the time scale of the project and cost effectiveness of the manufacture of the device. Therefore more consideration would be needed for the improved device into all of these factors and how best to incorporate them into a device that would perform better, meet the requirements of the user and solve the issues highlighted in this review of the current device.
6.5. Conclusions and Future work

Following the preliminary experiments using the current stretching device, several issues with the device became apparent and have been discussed in this Chapter. To improve on the current device, major changes would be required to improve the accuracy of the rod movements which controls the stretch exerted on the PDMS–RCE cell sample, to improve sample loading and unloading, to eliminate the need to move the RCE–PDMS sample from the device for imaging and to produce a device that could perform cyclic stretching and real-time imaging in one, compact design. For long term experiments, changes would need to include a system to maintain the environment within the device to ensure RCE cell survival and viability, providing temperature and gas control.

Further investigation into the effects of stretching on both the PDMS itself and the RCE cells cultured on PDMS is needed to improve the understanding of how an external mechanical stimulation can impact on certain cellular processes and how substrate stiffness and the extracellular environment of the cell respond to these changes. Molecular as well as physical and mechanical changes in the RCE cells need to be studied in more detail to provide more insight into the effects of stretching on the cell and its function. A key parameter of stretching that will be important in further experiments is the duration of stretch to investigate if longer stretch regimes have an effect on RCE cell morphology, orientation or migration. Also the strain exerted on the PDMS-RCE cells samples and how this affects RCE cell behaviour and also cyclic stretching parameters such as the rate of cyclic stretch on RCE cell behaviours such as cell alignment and migration will be critical in further understanding the RCE cell responses to mechanical stimulation.

Several methods have been used in the literature to measure and track substrate deformation by using beads embedded with the substrate. Traction force microscopy (TFM) is a useful technique for measuring the forces exerted by an object onto surfaces that the object adheres to or moves on. TFM uses an elastic substrate to which the object of interest is attached to and the deformations that are measured are converted into traction forces (Style et al., 2014). An important factor in TFM is to be able to accurately measure fluorescent or non-fluorescent beads that are used embedded in the substrate. TFM can be used to determine the deformation of a substrate and the displacement fields are determined using image analysis of the fluorescent beads within the substrate. Image processing could be done using software such as MATLAB to carry out the analysis on images obtained (Tseng et al., 2011). In terms of cell migration, TFM can be used to further understand the mechanics of cell migration and can transform bead movements that are embedded in a stiff polyacrylamide gel underneath a cell to mechanical stresses that have been induced by the contractile motion of the cell, driven by the cytoskeleton (Bloom et al., 2008). This technique allows...
accurate image correlation and tracking of beads embedded within the substrate. Through image processing, the deformation field of the substrate or displacement data can be extracted and de-convoluted to find out the cellular traction field using methods such as Fourier-Transform Traction Cytometry (FTTC) (Tseng et al., 2011; Schwarz and Soine, 2015). The types of beads used for this application were carboxylate polystyrene beads and some had been modified or were fluorescent (Bloom et al., 2008; Fraley et al., 2010; Tseng et al., 2011). The bead density used defines the spatial resolution and this can be determined in image processing methods used. Nanobeads were more favourable over microbeads in some applications as their main advantage is that they cause fewer disturbances to the mechanical properties of the substrate (Schwarz and Soine, 2015). Instead of polyacrylamide substrates, silicone-oil based substrates such as PDMS have also been used in TFM due to its excellent surface patterning properties. However it can also be a difficult material to handle in relation to adding in marker beads and the use of PDMS is therefore often done with 2 PDMS layers and a layer of marker beads that are placed between them (Schwarz and Soine, 2015). The different cell tracking methods discussed could improve image alignment and correlation between before and after stretch images, allowing the quantitative analysis of RCE cells and the effects of mechanical stimulation to be accurately tracked and to further understand the mechanisms involved.
Chapter 7
General Discussion, Conclusions, and Future Work
7.1. General Discussion and summary of work

The changes in the mechanical properties of cells and the interactions between the cell and the surrounding environment were found to be important in determining how cells respond to changes in the physical and mechanical environment. The mechanical response of cells is critical in understanding how particular cell processes are affected by tissue biomechanics, such as the wound healing response in the cornea and how the biomechanics of the cornea can affect the response to injury or surgery.

Single cell mechanical properties of rabbit corneal epithelial (RCE) cells were investigated in the work carried out in this thesis. Changes in substrate mechanical properties were used as a way of investigating the response of the RCE cells to changes in substrate stiffness. The substrates used were PDMS blends that were prepared so that they had a range of different Young’s moduli. The PDMS substrates were modified and treated with CAP oxygen plasma to prepare a more hydrophilic surface. This was shown to be successful in producing a more hydrophilic PDMS surface using water contact angle and XPS measurements to confirm changes in the surface chemistry following plasma treatment. The rate of hydrophobic recovery of the PDMS samples was also reduced by storing the samples in water. Different methods were used to measure the bulk mechanical properties of the PDMS blends and it was found that there was variation in the results obtained for Young’s modulus across the different testing methods used, which were tensile testing, ESPI and AFM. The general trend observed in the bulk mechanical properties tested by ESPI and AFM was that the Young’s modulus of the PDMS blends decreased as the ratio Sylgard 527 increased in the ratio of the two types used to prepare the PDMS blends (ratio of Sylgard 184: Sylgard 527).

These methods had greater sensitivity compared to tensile testing and it was therefore thought that this was the observed trend in the PDMS stiffness. However the values obtained from the bulk mechanical tests were much higher than those values reported in the literature for the stiffness of PDMS. It was reported that the tensile strength of PDMS ranged from 1-9 MPa (Schneider et al., 2008; Liu et al., 2009; Liu, Sun and Chen, 2009). In terms of the corneal biomechanical properties, the Young’s moduli, measured in vitro can range from 0.1–57 MPa in the literature (Elsheikh et al. 2008b; Garcia-Porta et al. 2014). Overall, Chapter 3 showed that PDMS substrates were prepared that had a range of different stiffness and that were successfully modified to produce a more attractive cell culture surface for testing the mechanical properties of the PDMS blends on the RCE cells.

Following this, Chapter 4 focused on the RCE cell work on the different PDMS blends. It was observed that as the Young’s modulus of the PDMS blends decreased, there were differences in the
RCE cell morphology and F-actin distribution. The F-actin staining images showed that on the softer PDMS blends there was less F-actin visible and therefore fewer stress fibres had formed. The presence of contractile stress fibres was thought to be critical for adhesion and maintaining cell attachment (Fusco et al., 2015). It was also shown that the RCE cell viability increased when cultured on the PDMS blends for 8 days in culture. However predicted cell numbers showed that the methods used meant that there was a discrepancy between the seeding density and the predicted number of cells on day 1 of culture. However, the viability assay confirmed that the proliferation and viability of RCE cells increased when cultured on the PDMS blends but was much lower that the tissue culture plastic control.

The RCE cell migration was also investigated on the PDMS blends by performing a scratch wound assay. The percentage wound closure was then calculated and it was observed that as the Young’s modulus decreased from PDMS 10:1 to PDMS 5:1, this correlated with a decrease in percentage wound closure over a 12 hour period. However this trend was not observed on PDMS 184 but all PDMS blend wound closure rates were lower than that of the tissue culture plastic control. Overall, Chapter 4 showed that the RCE cells could be successfully culture on the PDMS blends over different culture periods and showed that as the stiffness of the PDMS blend decreased, in particular on PDMS 1:1, there was a significant change in the RCE cell morphology and F-actin spreading and a slower wound closure rate compared to other PDMS blends and TCP. This showed that on the softer PDMS substrate the mechanical properties of the substrate affected the actin cytoskeleton interaction with adhesion molecules and also had an effect on the migratory mechanisms of the RCE cells.

The nanoscale mechanical properties of the RCE cell were then measured whilst cultured on the PDMS substrates using AFM. The overall trend observed was that the roughness of the cell decreased between the nucleus and the cell body whereas for Young’s modulus, it increased from nucleus to the cell edge. The results suggested that for a lower surface roughness, adhesion on a PDMS substrate was increased because of the increased stiffness of the RCE cell edge. The improved cell–matrix interactions may have correlated to cytoskeletal contractility whilst the cell was sensing mechanical changes in its environment. The cell–matrix adhesions tend to assemble initially at protruding cell edges and adhesion to the ECM is started through the lamellipodia and filopodia (Ridley 2003; Schwarz & Gardel 2012). This may have affected the Young’s modulus measurements for the cell edges. Overall, Chapter 5 showed that the RCE cell mechanics could be measured using the AFM and some significant differences were seen in particular that PDMS 184 was significantly
stiffer at the nucleus, next to nucleus and cell body regions but not at the cell edge. Further investigations would be required to further investigate the RCE cell mechanics at the nanoscale.

Lastly, Chapter 6 showed the preliminary data for an in-house mechanical stimulation device that could perform static uniaxial stretch on the PDMS–RCE cell samples. Overall the preliminary results did not show any differences in the RCE cell orientation or migration following static stress across three different strains for 15 minutes and 60 minutes. It was thought that these time periods were not long enough to see any change, as compared to the literature with some experiments being done over 24 hours, and that cyclic stretching or biaxial stretching would produce a greater response in the RCE cells (Gupta and Grande-Allen, 2006; Steward et al., 2010; Pang et al., 2011; Li et al., 2014). Further modifications to the device were needed and optimisation of the process was required.

7.2. Conclusions

In conclusion, the work shown in this thesis provided a method to successfully modify CAP plasma treated PDMS blends that had different Young’s moduli. It was observed that following CAP plasma treatment, the PDMS surfaces did increase in hydrophilicity and this produced a more attractive surface for cell adhesion. However, the mechanical testing of the PDMS blends did not all follow the hypothesised trend of decreasing PDMS stiffness with increasing amounts of Sylgard® 527. The methodology used for testing the mechanical properties of a substrate are dependent on what you are trying to measure and also the type of material being tested. Therefore, differences in the measurements can arise between the results obtained, showing the variability between different testing methods. Differences were observed across the PDMS blends in the RCE cell attachment, spreading and cell migration, in particular on the softer PDMS substrates PDMS 10:1 – 1:1, where cell responses in terms of RCE cell attachment and RCE cell migration decreased when compared to stiffer blends. These measurements also correlated with the AFM results in that the significant difference in Young’s modulus and roughness of the RCE cells were seen on the softer PDMS blends. However they did not always follow the expected trends and for the Young’s modulus of the cell edge, no obvious trend was observed. Based on the results obtained for the RCE cell mechanics at the cell edge, it was suggested that there could have been a correlation between the roughness of the PDMS surface and the Young’s modulus of the RCE cell edge that would have the most contact with the substrate surface and therefore be influenced more by changes in surface material and bulk mechanical properties of the PDMS blends.
The overall trend in the Young’s modulus data on RCE cell areas was that as the substrate stiffness decreased, so did the Young’s moduli measured for nucleus, next to nucleus and the cell body region of the RCE cell. The cell edge results were more variable, which was thought to be due to changes in the cytoskeletal structures within the cell, which is linked to cell adhesion behaviours. Across the different substrates and TCP, the cell edge stiffness increased compared to the other RCE cell areas. The preliminary results for the stretching of PDMS–RCE cell samples showed that the process required further optimisation and did not show any significant changes in cell alignment or migration towards the direction of the uniaxial stretch, as hypothesised.

The data showed a relationship between the PDMS sample stiffness and RCE cell response for cell attachment and migration, which indicated that the substrate properties had affected the cell adhesion molecules and the cytoskeletal network of actin filaments. This data related to the cell mechanics data in that the softer the substrate the lower the Young’s modulus or roughness for the particular cell area. However this trend was not a definitive trend observed across all conditions and more samples would be required to reduce the inherent biological variation across samples.

### 7.3. Future Work

Going forward from the work detailed in this thesis, firstly further work would be required for the bulk mechanical testing of the PDMS blends and the testing method would need to be standardised due to the large variation in the Young’s modulus data obtained using three different techniques. Once the methodology was decided, the protocol would remain the same in order to prepare a set range of Young’s moduli for the PDMS blends. For future studies on the Young’s modulus of substrates, the preparation parameters and their effects on the topography of substrates should be focused on along with stiffness measurements.

In terms of the PDMS preparation, the variation in the methodology could be reduced by using an automated mixer at a set speed and time. Also further tests could be carried out on the thickness measurements and how the mixing and curing parameters affect the thicknesses of the PDMS blends, which could impact on the bulk mechanical properties of the PDMS. Future work on plasma treatment could be investigated in terms of patterning of the PDMS surface with the plasma treatment and the effects of this on RCE cell alignment and migration. The scratch wound assay was an effective assay for investigating the wound closure rate of RCE cells cultured on the different PDMS blends but this could have been improved to perform a more accurate migration assay, for example using a silicone insert when seeding the RCE cells to form a zone of clearance rather a
scratch in the cell layer. This would create a gap with a defined width and without physically disrupting the cells (Kramer et al., 2013).

The cell adhesion of RCE cells was clearly affected by the softer substrates and future work on investigating these mechanisms further could be carried out using immunocytochemistry or antibody staining to fluorescently stain the RCE cells for adhesion molecules such as vinculin, which is a known component of focal adhesions and has a role in mechanotransduction. Furthermore, different cell types could be used within the system, moving onto human cells such as LESC in order to investigate the effects of mechanical stimulation on LESC migration and differentiation. The impact of this on the role of LESCs in the stem cell niche and in corneal and non-corneal tissue repair in the eye. The LESC have been reviewed in more detail in Chapter 1, Section 1.2.1.

The mechanical stimulation device showed no significant differences in the RCE cell after uniaxial static stretching. However the preliminary experiments provided more insight into the development of the device and the optimisation of processes used in RCE cell stimulation. This would allow the RCE cell mechanics to be linked to the impact of stress exerted onto the substrate or matrix surrounding the cell, and to investigate how the mechanical stimulation of cells affects behaviours such as cell migration and wound healing. This could have implications in the corneal tissue biomechanical response in that the substrates blends prepared could be fine-tuned to mimic physiological stiffness representative of the cornea following injury or in disease states.

The impact of the uniaxial stretch on the orientation and movement of RCE cells needs to be interpreted with further understanding of the mechanical behaviours of PDMS under tensile load. Also the macroscopic mechanical interactions would need to be extended to the microscopic or cellular scale interactions. However a longer duration of stretching and different parameters such as the ability to perform cyclic stretching and finer movements improving the control of the stretch would allow more information on the responses of RCE cells to be extracted from the data obtained. Lessons learnt from the preliminary device will enable the future design of a more accurate, compact and online stretching system incorporating the successfully cultured RCE cell–PDMS samples and changes have been considered after critical analysis of the current device, for future work going forward. The methods used involving bead markers for cell tracking, as discussed in Chapter 6, could be applied in further experiments of mechanical stimulation of PDMS samples to be able to use image processing software for image correlation and correct alignment of samples. This would enable RCE cell tracking and also the ability to measure cell-induced displacement of beads. Furthermore, image analysis could be carried out as batch processes using software such as MATLAB.
Application of these methods would improve experimental work allowing RCE cell changes to mechanical stimulation to be distinguished from material deformations.

The preliminary device runs highlighted several issues and modifications that are necessary for future device improvements and allowed a proof-of-concept stretching device to be successfully run for living cells. Although the results did not show significant changes in the RCE cells after stretch for all conditions tested, the data allowed the initial parameters and settings of the device to be tested on successfully loaded RCE cell–PDMS samples and live cell imaging to be carried out offline.
Chapter 8

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