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The Role of the Actin Cytoskeleton in the Response of Chondrocytes to Mechanical Loading: A Computational and Experimental Investigation

by


A thesis submitted to the National University of Ireland as fulfilment of the requirements for the Degree of Doctor of Philosophy

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Abstract

The biomechanisms which govern the response of chondrocytes to mechanical stimuli are poorly understood. This thesis aims to provide a more in depth understanding of chondrocyte biomechanics by focusing on the role of actin cytoskeleton in the response of chondrocytes to mechanical loading. Novel single cell in vitro experiments in tandem with finite element simulations using a 3D active modelling formulation, incorporating actin cytoskeleton remodelling and contractility, are performed.

Experimentally, single chondrocytes are subjected to shear deformation by a horizontally moving probe. Untreated chondrocytes containing a contractile actin cytoskeleton exhibit a distinctive force-indentation curve whereby force increases rapidly upon initial probe contact, followed by a yield point and a reduced rate of force increase. In contrast, cells in which the actin cytoskeleton has been disrupted exhibit a linear force-indentation curve, with measured forces being significantly lower that for untreated cells. Simulations using the active 3D framework reveal that the distinctive response of untreated cells to applied shear is due to the yielding of the actin cytoskeleton in tensile regions of the cell and dissociation of the actin cytoskeleton in compressive regions of the cell. In contrast, a simple passive hyperelastic model is sufficient to predict the linear force-indentation curve for cells in which the actin cytoskeleton has been disrupted. Disruption of intermediate filaments and microtubules did not alter the distinctive force-indentation curve observed for untreated cells, further highlighting the critical role of the actin cytoskeleton in chondrocyte biomechanics.
The 3D active modelling framework is also implemented to investigate the increased probe force required to detach spread chondrocytes from a flat substrate. Simulations reveal that spread cells with a flattened morphology have a more highly developed actin cytoskeleton than rounded cells. Rounded cells provide less support for tension generated by the actin cytoskeleton, hence a high level of dissociation is predicted. As a result of the higher level of actin cytoskeleton in the cytoplasm of spread cells, significantly higher detachment forces are computed than for round cells, as observed in the experimental study of Huang et al. (2003).

Furthermore, the biomechanical response of chondrocytes in situ, and in particular, the response of the actin cytoskeleton to physiological and abnormal strain loading is investigated. Simulations predict that the presence of a focal defect significantly affects cellular deformation, increases the stress experienced by the nucleus, and alters the distribution of the actin cytoskeleton. It is also demonstrated that during dynamic loading, cyclic tension reduction in the cytoplasm leads to continuous dissociation of the actin cytoskeleton. In contrast, significant changes in cytoplasm tension are not predicted during static loading, and hence the rate of dissociation of the actin cytoskeleton is reduced.

The combined modelling-experimental approach presented in this thesis provides new insight into the role of the active contractility and remodelling of the actin cytoskeleton in the response of chondrocytes to mechanical loading. The findings of this thesis may have important implications for understanding the mechanisms involved in the pathogenesis of cartilage tissue and for tissue engineering of cartilage.
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1 Introduction

1.1 Background

Articular cartilage is a load bearing material that is found lining diarthrodial joints in the body such as the knee, hip and finger joints. It is a specialised type of hyaline cartilage which has important biomechanical functions to serve in order to maintain joint equilibrium such as allowing frictionless motion of articulating joints and acting as a lubricant between bones that would otherwise be in contact. Significantly, articular cartilage also functions in resisting and distributing loads from one bone to another (Buckwalter et al. 1991; Mow and Ratcliffe 1997).

External loads that are applied to articular cartilage are conveyed through the extracellular matrix down to the most basic level of the tissue, the chondrocyte. The chondrocyte is structurally supported by several important elements which comprise the cytoskeleton, namely actin filaments, microtubules and intermediate filaments. These structural elements play a role in helping the chondrocyte to withstand the various loads that occur during everyday activity such as compression, tension, shear and hydrostatic pressure (Ofek and Athanasiou 2007; Blain 2009). Experimentally it has been shown that extreme loads can alter the transmission of signals within the cell or the nucleus, resulting in changes in gene expression and decreased ECM synthesis (Jeffrey et al. 1995; Kurz et al. 2001; Quinn et al. 2001; Lee et al. 2005). Additionally, it has been demonstrated that the cytoskeleton is linked to cartilage homeostasis and pathology (Capin-Gutierrez et al. 2004; Fioravanti et al. 2005; Lyman et al. 2012). Significantly, cartilage tissue is deficient of blood vessels and a lymphatic system, and lacks high numbers of cells. As a result, cartilage has a limited repair capacity. When cartilage is damaged, either due to abnormal loading conditions or trauma, there is an increase in chondrocyte apoptosis and inadequate synthesis of new extracellular matrix. This can ultimately lead to the onset of osteoarthritis (Figure 1.1). Millions of people worldwide suffer with this disease, and it is thought to be the most common chronic joint disease (Bijlsma et al. 2011).

In order to develop effective treatment methodologies for diseases such as osteoarthritis, firstly, it is necessary to understand the effect of mechanical forces in
cartilage homeostasis. One current approach is to focus on the fundamental unit of cartilage tissue, the chondrocyte. The biomechanical behaviour of chondrocytes has been experimentally investigated using a number of high-precision techniques including single cell compression and shear devices, atomic force microscopy, micropipette aspiration, as well chondrocyte / agarose gel culture systems. Numerous in vitro studies have demonstrated that chondrocytes respond to mechanical stimuli (Guilak et al. 1999; Jones et al. 1999; Huang et al. 2003; Darling et al. 2006; Shieh and Athanasiou 2007; Leipzig and Athanasiou 2008). Additionally, the role of the cytoskeleton in response to externally applied loading has been studied, using fluorescent imaging techniques (Knight et al. 2006; Campbell et al. 2007) or through the use of chemical agents that disrupt individual cytoskeletal components (Trickey et al. 2004; Ofek et al. 2009b). Despite such extensive in vitro investigation, the mechanisms by which chondrocytes sense and react to mechanical loading are poorly understood.

Computational modelling has also been used to investigate the biomechanics of chondrocytes. A number of models such as linear elastic, viscoelastic and biphasic models have been employed to simulate the aforementioned experimental techniques (Guilak and Mow 2000; Guilak et al. 2000; Baaijens et al. 2005; Trickey et al. 2006; Vaziri and Mofrad 2007; Kim et al. 2008; Ofek et al. 2009a). Typically, these models have been used to determine the mechanical properties of individual chondrocytes. However, it has been demonstrated that passive viscoelastic models can only capture experimental behaviour by artificially modifying cell material properties for different cell geometries (McGarry and McHugh 2008). The use of such passive visco/hyper-elastic material models ignore the key mechanisms by which cells respond to mechanical stimuli, and hence offer a limited insight into chondrocyte mechanotransduction.

1.2 Motivation

The focus of this thesis is on the role of actin cytoskeleton in the response of chondrocytes to mechanical loading. Previous studies have investigated how cells respond to mechanical stimuli in terms of mechanical behaviour, gene expression and remodelling of the actin cytoskeleton. The following experimental investigations have provided motivation for this project:
Cyclic compression of chondrocytes has shown to upregulate type II collagen and aggrecan while static compression down-regulated those particular genes (Shieh and Athanasiou 2007).

Compression of chondrocytes in agarose gel leads to disruption of the actin cytoskeleton (Knight et al. 2006; Campbell et al. 2007).

Treatment of chondrocytes with growth factors followed by dynamic compression has been shown to have a synergistic effect in elevating levels of type II collagen (Mauck et al. 2003).

These studies demonstrate that chondrocytes react to mechanical stimuli and that the actin cytoskeleton plays a role in this response. However, the connection between the mechanical loading of chondrocytes, remodelling of the actin cytoskeleton, and nucleus deformation is poorly understood.

1.3 Overall Goal and Specific Objectives of the Thesis

The overall goal of this thesis is to gain a more in depth understanding of the role of the actin cytoskeleton in chondrocyte biomechanics by performing novel single cell experiments and computational simulations. The specific objectives of this thesis are as follows:

- Experimental investigation of the role of the cytoskeleton in single chondrocytes in response to shear deformation in vitro

- Implementation of an active biomechanical model incorporating cellular contractility to simulate mechanically sheared chondrocytes and subsequent validation with experimental data
Chapter 1

- Computational investigation of the effect of the active contractility and remodelling of the actin cytoskeleton on the resistance of spread chondrocytes to detachment

- Computational investigation of in situ chondrocyte deformation and actin cytoskeleton remodelling during physiological loading

Figure 1.2 shows a flowchart depicting the overall structure of this thesis. Firstly, in vitro experiments are performed to study the effect of the cytoskeleton on the shear resistance of single chondrocytes. Next, the ability of the active modelling formulation to capture the distinctive behaviour observed in the in vitro experiments is demonstrated. The active model is then implemented to simulate single chondrocyte detachment and in situ chondrocyte deformation, focusing on the role of the actin cytoskeleton.

1.4 Thesis Overview

This section contains a brief description of each chapter of thesis. In Chapter 1, the present chapter, an introduction to the research topic and an overview of the thesis is provided. Additionally, the objectives of the thesis are stated. Chapter 2 presents a review of the current literature, including a description of the structure of articular cartilage, chondrocytes, and the cytoskeleton. Experimental methods which have been designed to investigate the biomechanics of chondrocytes are also reviewed. Furthermore, studies using computational modelling techniques to simulate chondrocyte and cartilage tissue biomechanical behaviour are presented. The active modelling framework implemented in this thesis and necessary background information is presented in Chapter 4. Literature that is directly relevant to the studies performed in this thesis (Chapters 5, 6 and 7) is presented at the start of those chapters, as distinct from the more general background literature on chondrocyte and cartilage biomechanics presented in Chapter 2.

Chapter 3 presents the experimental methods that are carried out in this work. The harvesting and isolation of single chondrocytes from bovine articular cartilage is described. The experimental test-rig used to apply a shear deformation on
cells and associated protocols are then presented. A novel video-capture method developed in order to visualise the morphological alterations of single chondrocytes during shear is described. Precise measurements of force and probe indentation may be acquired using this technique. Finally, a novel method developed to visualise the intracellular organisation of the actin cytoskeleton, focal adhesions, and the nucleus during mechanical shear is described.

In Chapter 4, a review of the fundamental mathematical theory relating to the computational work in this thesis is presented. Firstly, a brief introduction is given to continuum mechanics theory, followed by a synopsis of hyperelastic and anisotropic hyperelastic material behaviour. An active modelling framework which incorporates the biomechanisms underlying the formation, dissociation and contractility of the actin cytoskeleton is used to represent the chondrocyte cytoplasm in this work. Therefore, a description of this active bio-chemo-mechanical model, including the numerical implementation is provided.

Chapter 5 presents an experimental and computational investigation into the effect of remodelling and contractility of the actin cytoskeleton on the shear resistance of single cells. In this study, a series of in vitro experiments are performed, in which single chondrocytes are subjected to shear deformation by a horizontally moving probe. Additionally, experiments are also performed on chondrocytes in which the three components of the cytoskeleton have been disrupted. The active model introduced in Chapter 4 is implemented in order to simulate the in vitro shear experiments. Validation of the active model for single chondrocytes is achieved by accurately simulating the biomechanical response of chondrocytes to applied shear.

Chapter 6 presents a computational follow-on study to Chapter 5 in which the shear detachment response of single chondrocytes is investigated. Finite element meshes of round and spread chondrocytes are created based on experimentally measured cell geometries. A cohesive zone model is used in order to simulate debonding at the cell-substrate interface.

The biomechanical behaviour of chondrocytes in situ in response to physiological loading conditions is investigated in Chapter 7. A 3D unit cell for
cartilage tissue is created, comprising of a chondrocyte, the pericellular matrix, and the extracellular matrix. Again, the active modelling framework is implemented to predict the biomechanical behaviour of chondrocytes. The effect of the focal defects, removal of the pericellular matrix, and tissue anisotropy is also examined.

Further discussion and concluding remarks are presented in Chapter 8.
References


Figures

Figure 1.1 Comparison of (a) normal (healthy) and (b) osteoarthritic human cartilage tissue. Reproduced from Han et al. (2011). *Figure blurred due to copyright reasons.*
Figure 1.2 Flowchart depicting the structure of this thesis. The experimental methods and results for the *in vitro* investigation of single chondrocytes during shear deformation are presented in Chapters 3 and 5 respectively. The theoretical framework, and the implementation and validation of the active model are presented in Chapters 4 and 5 respectively. The active model is then implemented to investigate the effect of the actin cytoskeleton during chondrocyte detachment in Chapter 6, and *in situ* chondrocyte deformation in Chapter 7.
2 Literature Review

2.1 Structure and Function of Articular Cartilage

2.1.1 Introduction

Joints in the human body, such as the knee and hip joint, are subjected to a complex combination of compressive, tensile, shear and hydrostatic stresses during normal movement. Normal adult cadence corresponds to 0.6 - 1.1 Hz loading per leg and greater than 1.5 Hz during running, which can cause joints to experience contact stresses up to 20 MPa (Morrell et al. 2005). This can result in cartilage tissue deformation levels of approximately 5 - 10% (Eckstein et al. 2000; Kersting et al. 2005). Cartilage also exhibits excellent wear resistance and lubrication properties, with studies showing that friction coefficients are in the range of 0.0005 – 0.04 (Forster and Fisher 1996; Tanaka et al. 2004; Ateshian and Mow 2005). The biomechanical function of articular cartilage is closely related to the zonal architecture of the tissue.

2.1.2 Extracellular Matrix

The extracellular matrix (ECM) of articular cartilage exhibits non-linear, time-dependent, anisotropic, and inhomogeneous mechanical behaviour due to the non-uniformity of its structure. Cartilage explant studies have shown that strain fields in the tissue vary according to depth and location (Wong and Sah 2010). It consists of four separate zones, namely the superficial tangential zone, the middle zone, the deep zone and the calcified layer zone (Figure 2.1). Each zone contains a varying matrix and cellular composition, and thus exhibits different mechanical properties. Despite the difference in the structure of each layer, a relatively simple number of components form the matrix of articular cartilage. Interstitial water is the main component, accounting for 60-85% of the wet weight of the tissue. The solid phase of articular cartilage is comprised of collagens and proteoglycans, with 50-75% of the tissue composed of collagens and the rest being proteoglycans. Collagens are responsible for the tensile characteristics of the tissue due to the formation of crosslinking fibres (in particular collagen type II). Proteoglycans such as aggrecan are chains of long, unbranched, glycosaminoglycans (GAGS) attached to a central...
protein which contributes to the compressive properties of cartilage (Buckwalter et al. 1991; Mow and Ratcliffe 1997; Koay and Athanasiou 2009).

The superficial zone (10-20% of the thickness of the tissue) is composed primarily of collagen fibres, while there is a low amount of proteoglycans. The superficial zone is significant for its tangential alignment of collagen fibrils and high water content thus promoting high tensile properties. Chondrocytes in the superficial zone appear flattened and elongated, and are grouped together in horizontal clusters at an average angle of 20° with respect to the articular surface (Jadin et al. 2007). In the middle zone (60% of the thickness of the tissue), the collagen fibres are arranged randomly. Additionally, the water content is decreased and chondrocytes are of a more rounded morphology. Furthermore, the largest numbers of proteoglycans are found in the middle layers of the issue. The deep zone contains the lowest volume of water and collagen compared to the other zones. Collagen fibres are aligned perpendicular to the articular surface, resulting in this region having high compressive mechanical properties (Mow and Ratcliffe 1997). Chondrocytes are orientated perpendicular to the articular surface, appear ellipsoid in shape, and are organised into vertical columns. Cellularity is greatest in the superficial zone with approximately 150x10^6 cells per cm^3, and decreases in the middle and deep zones with approximately 50x10^6 cells per cm^3 at a tissue depth of 1 mm (Jadin et al. 2005). The calcified zone is below the deep zone, leading to the subchondral bone. The deep and calcified zones are separated by a narrow band of minerals called the tidemark.

2.1.3 Pericellular Matrix

The pericellular matrix (PCM) is a narrow band of tissue that surrounds a chondrocyte cell within the ECM, and together they represent the chondron. The PCM contains a higher concentration of proteoglycans (aggrecan, hyaluronan, and decorin) than the ECM, and also contains collagens II, VI and IX (Poole 1997). Importantly, in articular cartilage collagen VI is only found in the PCM. Chondrocytes are attached to collagen VI fibres through integrin receptors (Doane et al. 1998), and it is hypothesised that collagen VI helps to anchor the chondrocyte to the PCM (Marcelino and McDevitt 1995). Hence, it is likely that the PCM acts as a transducer of biochemical and biophysical signals that are transferred from the ECM.
to the chondrocyte (Guilak et al. 2006). Numerous experimental studies have investigated the biomechanical role of the PCM. It is widely believed that the PCM protects chondrocytes from stress associated with mechanical joint loading (Poole et al. 1984; Poole 1997). Knight et al. (1998b) has shown that chondrocytes surrounded by an intact PCM were more resistant to deformation compared to chondrocytes with a partially degraded PCM. In addition, changes in the structure of the PCM have been shown to modify chondrocyte shape in osteoarthritic cartilage (Korhonen et al. 2011). In terms of mechanical properties, the Young’s modulus of the PCM is approximately an order of magnitude greater than the Young’s modulus of chondrocytes, and 1-2 orders lower than the ECM (Alexopoulos et al. 2003; Guilak et al. 2005).

### 2.2 Chondrocytes

#### 2.2.1 Overview

Mature differentiated chondrocytes occupy approximately 10% of articular cartilage and produce ECM molecules necessary for healthy viable tissue such as collagen type II and aggrecan. Although chondrocytes have a limited capacity for replication, there is sufficient matrix production and secretion of molecules during normal activities. However this becomes a significant factor when injury or trauma occurs, as there is only a limited increase in matrix synthesis. In order to understand the mechanisms involved in the cause of cartilage related diseases, it is necessary to study how mechanical stimuli are conveyed from the tissue to subcellular levels. The main biomechanical constituents of chondrocytes, the cytoskeleton and the nucleus, are discussed in the following subsections.

#### 2.2.2 Cytoskeleton

The cytoskeleton of chondrocytes functions in maintaining the structure of the cell, plays a crucial role in defining the mechanical properties of the cell, and transmits mechanical signals from the cell membrane to the nucleus (Ingber 1997; Shieh and Athanasiou 2003; Wang and Thampatty 2006; Bader and Knight 2008; Fletcher and Mullins 2010). Each component of the cytoskeleton is composed of proteins that are arranged in an organised and specific pattern. The three main
components of the cytoskeleton are: actin filaments, intermediate filaments, and microtubules (Figure 2.2).

Actin is a globular protein with an ATP binding site that serves as a site for nucleation of G-actin monomers and for the growth of the actin filaments (Figure 2.3). G-actin forms F-actin in the presence of ATP, Mg and K. Actin filaments are composed of two threaded polymer chains of F-actin, with each filament 5 – 9 nm in diameter. It is known that actin microfilaments are essential for functions such as cell motility, morphology, adhesion and contractility (Guilak et al. 1995; Burridge and Chrzanowska-Wodnicka 1996; Alberts et al. 2002; Becker et al. 2003; Trickey et al. 2004; Woods et al. 2007; Blain 2009; Fletcher and Mullins 2010). Actin filaments also play a critical role in maintaining the chondrocyte phenotype (Woods et al. 2007). An important feature of actin filaments is their capability to interact with myosin to form tightly woven bundles of ‘stress fibres’. In a suspended or resting state, the cytoplasm contains short actin filaments that are capped by the protein CapZ and myosin II is present in an inactive state, thereby preventing any interaction between actin and myosin. In response to an activation signal, several intracellular pathways (such as Rac, Rho, and Cdc42) stimulate actin filament growth and myosin II activation. The signal results in a large influx of calcium ions (Ca$^{2+}$) in the cell cytosol, which leads to the activation of gelsolin. Gelsolin cleaves capped actin filaments into small fragments. The process of severing and uncapping by gelsolin leads to formation of long filaments. Actin filaments are then bundled together by either α-actin or fimbrin. α-actin loosely bundles actin filaments together, allowing myosin to enter and form contractile stress fibres. Fimbrin, however, causes actin filaments to be tightly bundled and prevents any interaction with myosin (Alberts et al. 2002; Blain 2009).

Phosphorylation of Myosin II by light-chain-kinase in response to a rise in calcium ions in the cytosol, or by Rho-kinase due to an externally applied signal causes myosin II to change from a bent state to an extended state. Myosin then assembles into bipolar filaments and interacts with actin filaments that are bundled together by α-actin, resulting in the formation of stress fibres. Contractile forces are generated via a cross-bridge cycling process, similar to that observed for skeletal muscle whereby the hydrolysis of ATP causes the myosin head to pivot and exert
traction on the attached actin filament (Figure 2.4). When a stress fibre is fully activated, there is no net sliding between actin and myosin, and the maximum amount of myosin heads are attached to actin filaments. In this scenario, the stress fibre experiences an isometric tension. If tension decreases or the contraction/shortening rate of the fibres is increased, the number of myosin heads attached to actin filaments will decrease. This will lead to the dissociation of stress fibres (Alberts et al. 2002). In the cytoplasm, stress fibres connect with integrins at focal adhesion sites, which attach to ligands in the ECM. This creates a link between the cytoskeleton and the ECM, allowing the cell to respond to external stimuli and elicit a response such as exerting a contractile force on the cell’s surroundings (Burridge and Chrzanowska-Wodnicka 1996).

Intermediate filaments have a diameter of 10 nm and are primarily composed of vimentin in chondrocytes. They are composed of a series of proteins tetramers, which are formed from two coiled dimmers, and arranged in an anti-parallel manner. The tetramers then assemble to form a super-coiled sheet similar to a rope-like appearance (Fuchs and Weber 1994; Blain 2009). Intermediate filaments are known to be the most stable of cytoskeletal elements and are believed to be important in mechanically linking the nuclear lamina with integrin receptors on the surface (Alberts et al. 2002; Becker et al. 2003). The precise functions of intermediate filaments is poorly understood but it has been shown that they play a role in biochemical responses (Wang et al. 1993) and resisting mechanical deformation (Ingber 1997; Durrant et al. 1999; Ofek et al. 2009b).

Microtubules are formed in the centrosome of the cell and consist of hollow cylindrical tubes of the globular protein tubulin. Microtubules are arranged linearly into protofilaments that form cylindrical structures, and are the largest fibre-like structure of the cytoskeleton, with a diameter of 25 nm. Microtubules are involved in important cell processes such as cell division, motility, and the transport of organelles (Alberts et al. 2002; Blain 2009). Additionally, it has been shown that tubulin is important for the synthesis and secretion of proteoglycans and collagen (Poole et al. 2001). From a cell mechanics perspective, an in vitro study by Ofek et al. (2009b) has shown that microtubules play a role in resisting mechanical deformation in chondrocytes. However, a study by Trickey at al. (2004) reported that
disrupting the microtubule network had no effect on the stiffness and viscosity of the cell.

The structure of the major cytoskeletal elements may be manipulated with the use of chemical agents (Figure 2.5). Cytochalasin-B/cytochalasin-D is commonly used to disrupt actin microfilaments and preventing stress fibres from forming (Schliwa 1982). Cytochalasin D binds to the (+) end of F-actin and prevents further addition of G actin monomers. The chemical colchicine or nocodazole disassembles the microtubule network in the cytoplasm and acrylamide has a similar effect on intermediate filaments.

2.2.3 Organisation of the Actin Cytoskeleton In Situ and In Vitro

This thesis focuses primarily on the role of the actin cytoskeleton chondrocytes. Therefore it is necessary to provide a description of the organisation and arrangement of the actin cytoskeleton in chondrocytes, as reported in the literature. The organisation of the actin cytoskeleton has been characterised in situ in cartilage explants (Durrant et al. 1999; Langelier et al. 2000), in primary chondrocytes cultured in a ‘3D environment’ such as agarose or in suspension (Idowu et al. 2000; Knight et al. 2001a; Trickey et al. 2004; Knight et al. 2006b; Campbell et al. 2007; Haudenschild et al. 2009; Haudenschild et al. 2011; Chen et al. 2012; Pravincumar et al. 2012), and also in chondrocytes adhered to substrates in vitro in a ‘2D environment’ (Brown and Benya 1988; Mallein-Gerin et al. 1991; Huang et al. 2003; Woods et al. 2005; Leipzig et al. 2006; Campbell et al. 2007; Campbell and Knight 2007; Leipzig and Athanasiou 2008; Haudenschild et al. 2009; Ofek et al. 2009b).

Durrant et al. (1999) examined the actin cytoskeleton in chondrocytes within their ECM that were dissected from adult rat femoral heads. The study demonstrated that bright foci of actin were located at the cell surface, and were linked to an irregular actin network extending inwards beneath the cell membrane (Figure 2.6 (a)). The study by Langelier et al. (2000) investigated the structure of the actin cytoskeleton in intact sections of mature bovine articular cartilage, and demonstrated that actin was dense, punctate, and cortically arranged (Figure 2.6 (b)). Additionally,
studies have shown that the arrangement of the actin cytoskeleton does not vary from zone to zone in articular cartilage (Durrant et al. 1999; Langelier et al. 2000).

Actin cytoskeleton organisation has also been quantitatively and qualitatively characterised in isolated chondrocytes that have been cultured in agarose gel. Knight et al. (2001a) showed that the greatest level of actin organisation was found to be around the periphery of the cell, and that actin arrangement was not a function of culture time (7 days) (Figure 2.7 (a)). In addition, chondrocytes cultured in 4% agarose gel for three days contained punctate actin filaments that were organised as a 1-2 μm thick cortical shell at the cell membrane (Knight et al. 2006b) (Figure 2.7 (b)). Furthermore, in the study of Idowu et al. (2000), chondrocytes seeded within 6% agarose gel contained a non-organised actin cytoskeleton after one day of culture, with no clearly defined cortical actin network observed. After seven days of culture, the actin cytoskeleton was clearly organised, with cortical actin observed at the cell periphery (Figure 2.7 (c)). Trickey et al. (2004) have demonstrated that human chondrocytes suspended in 1.2% alginate beads in media exhibit a clear solid ring of F-actin around the cell cortex (Figure 2.8 (a)). Recently, chondrocytes in suspension (Figure 2.8 (b)) (Pravincumar et al. 2012), and embedded in agarose gel (Figure 2.8 (c)) (Chen et al. 2012) and 2% alginate (Figure 2.8 (d)) (Haudenschild et al. 2011) have been shown to have a distinct ring of cortical actin.

In vitro, studies have demonstrated that for chondrocytes seeded on stiff glass substrates for a time period of approximately three hours, the actin cytoskeleton is quite smeared in appearance, with no distinct ring of cortical actin evident (Figure 2.9) (Leipzig and Athanasiou 2008; Ofek et al. 2009b). Continued culturing of chondrocytes on substrates permits cell spreading, and this has been shown to result in de-differentiation to a fibroblastic-type phenotype. This process is characterised by the formation of highly aligned stress fibre bundles (Figure 2.10) (Brown and Benya 1988; Woods et al. 2005; Campbell et al. 2007; Haudenschild et al. 2009; Chen et al. 2012), which are not found in chondrocytes in situ.

2.2.4 Nucleus

The nucleus of the chondrocyte contains its own membrane (nuclear envelope) and cytoskeleton (nuclear lamina). The chromatin contained in the nucleus
is linked to the cytoskeleton in the cytoplasm via these nuclear structures. Deformation of the nucleus is regarded as one of the most direct transducers of cellular mechanotransduction in chondrocytes (Guilak 1995; Shieh and Athanasiou 2003). The response of the nucleus to mechanical stimuli in terms of changes in its morphology and volumetric characteristics may lead to changes in chromosome alignment and subsequently gene expression (Buschmann et al. 1996; Leipzig and Athanasiou 2008).

In relation to the mechanical properties of the chondroctye nucleus, there are contrasting reports found in literature, particularly regarding the stiffness of the structure. It has been shown that the isolated nuclei are 3 – 4 times stiffer than the cytoplasm (Guilak et al. 2000). Similar studies for isolated nuclei of endothelial cells have been reported also (Caille et al. 2002). In contrast, a study by Leipzig and Athanasiou (2008) found that cellular and nucleus strains had a 1:1 ratio when compressed in situ (Leipzig and Athanasiou 2008). However, the properties of the nucleus will be inherently affected by their surrounding environment, whether isolated or in situ, and by the testing modality.

2.3 Mechanical Stimulation of Chondrocytes

2.3.1 Overview

The mechanisms in which chondrocytes respond to mechanical stimuli are poorly understood. There is a need for a greater knowledge of these mechanisms in order to gain a firmer understanding of cartilage mechanics. The majority of previous studies into these mechanisms have focused on a large population of cells, for example, cartilage explants (Sah et al. 1989; Kim et al. 1994; Buschmann et al. 1995; Guilak 1995; Frank et al. 2000; Jin et al. 2001; Li et al. 2001; Quinn et al. 2001; Jin et al. 2003; Lee et al. 2005) and 3D tissue constructs (Buschmann et al. 1995; Lee et al. 1998; Vunjak-Novakovic et al. 1999; Mauck et al. 2000; Gooch et al. 2001; Shahin and Doran 2012). Although this is a necessary step in order to gain knowledge about how chondrocytes respond to mechanical loading when located in their native ECM, an alternative approach is to explore the specific effect of stimuli on single cells (Shieh and Athanasiou 2003). This approach allows researchers to obtain insight into the behaviour of the fundamental unit of articular cartilage.
Testing chondrocytes on the tissue scale fails to take into account important variables such as cell morphology, and remodelling and contractility of the actin cytoskeleton. Additionally, chondrocytes within the same population may experience different stresses and strains and the response of the whole population of cells may not be an accurate reflection of each cell.

The advantage of the single cell approach is that it allows the researcher to observe the resulting changes in cellular behaviour after the application of particular mechanical stimuli. A relationship may then be developed which explains the effect that the stimuli has on the cell. This may include examining mechanical properties, gene expression and intracellular structural changes such as remodelling of the actin cytoskeleton. Furthermore, the investigation of osteoarthritic chondrocytes and subsequent comparison to the behaviour of healthy chondrocytes may shed light on the pathways involved during the onset of osteoarthritis (Ofek and Athanasiou 2007). The single cell approach is not without its limitations. Non-confluent cells seeded on to a glass slide or petri dish do not represent the in vivo environment, nor does it represent the typical tissue engineering environment of cells seeded on to a scaffold. Consequently, the response of those individual cells can be potentially different than cells located in a three dimensional scaffold as the cells are not directly loaded by the mechanical stimuli (Shieh and Athanasiou 2003). However, studies on a single cell level can be employed to explore the type of mechanical environment necessary to elicit favourable cellular responses that will promote the production of essential ECM components for cells seeded in tissue constructs (Ofek and Athanasiou 2007).

The type of equipment needed to measure forces and deformations on the single cell level must be extremely sensitive, easy to manipulate and be accurate down to the nano-scale. Several techniques have been developed in the last 15 years that meet the aforementioned criteria, allowing investigators to study the behaviour of cells in response to mechanical stimulation (Figure 2.11). The following section describes the most common techniques used for the mechanical testing of cells, primarily concentrating on experiments preformed with chondrocytes, with selected references made to experiments involving other cell phenotypes.
2.3.2 Compression

Many cell types, including chondrocytes, experience high levels of compression during everyday activity. Several experimental techniques have been developed in order to examine the effect of whole-cell compression on in vitro chondrocyte behaviour. The load imparted on the cell may be either force-controlled (Koay et al. 2003; Leipzig and Athanasiou 2005; Shieh and Athanasiou 2007; Leipzig and Athanasiou 2008) or strain-controlled (Shieh et al. 2006; Koay et al. 2008; Ofek et al. 2009b). This method involves using a flat-ended probe that is larger than the size of the cell to perform indentation (Figure 2.12). A piezoelectric translator drives the probe a prescribed distance, and cantilever beam theory is used to determine the applied force based on the deflection of the beam. Static, intermittent, and dynamic compression may be chosen as the loading condition. Single cell compression experiments have been used in combination with PCR methods in order to quantify gene expression within a mechanically deformed cell (Eleswarapu et al. 2007). It was found that levels of cartilage regenerative related genes (collagen type II and aggregan) decrease during static compression (50 and 100 nN) whereas dynamic loading results in an up-regulation (Shieh and Athanasiou 2007). The effect of compression on the chondrocyte cytoskeleton and the nucleus has also been investigated using the aforementioned technique (Leipzig and Athanasiou 2008; Ofek et al. 2009b). Ofek et al. (2009b) demonstrated that disruption of the actin cytoskeleton results in a significant reduction in the stiffness of chondrocytes compared to the disruption of intermediate filaments and microtubules. Taken together, these studies suggest that particular loading modes lead to intracellular structural changes, which have a profound effect on the gene expression of the cell.

Compressive loading of chondrocytes embedded in agarose gel has provided novel insight into chondrocyte behaviour in a 3D environment (Knight et al. 1998a; Lee et al. 1998; Idowu et al. 2000; Lee et al. 2000; Knight et al. 2001b; Knight et al. 2002; Knight et al. 2006a; Knight et al. 2006b). In particular, compression of chondrocytes in agarose gel has been shown to induce disruption of the actin cytoskeleton (Knight et al. 2006b; Campbell et al. 2007). Additionally, this system has been used to study the relationship between intracellular calcium signalling and...
compressive loading of chondrocytes (Roberts et al. 2001; Pingguan-Murphy et al. 2005; Pingguan-Murphy et al. 2006).

2.3.3 Shear

Shear deformation has been shown to affect chondrocyte metabolism in a monolayer (Smith et al. 2000), and chondrocyte morphology in agarose constructs (Sawae et al. 2004). On a single cell level, shear loading has been used to examine cellular behaviour and to measure the force required to detach cells from a substrate. Similar to single cell compression experiments, cantilever beam theory is typically used to calculate the reaction force of the probe based on the deflection imparted by the cell. Athanasiou et al. (1999) reported that a greater force was required to detach primary chondrocytes that were seeded on fibronectin compared to cells attached to bovine serum albumin and on untreated glass. Similarly, it has been shown that single fibroblasts have a higher adhesion strength when attached to collagen-coated polystyrene compared to glass (Yamamoto et al. 1998; Yamamoto et al. 2000). Furthermore, in vitro studies have shown that single chondrocytes become more resistant to shear forces as the cell spreads and the actin cytoskeleton reorganises to the periphery of the cell (Hoben et al. 2002; Huang et al. 2003). The aforementioned experiments have particular relevance for understanding cell adhesion and the relationship between the cell-substrate interface in order to design scaffolds for the tissue engineering of cartilage constructs.

2.3.4 Atomic Force Microscopy / Nano-indentation

Atomic force microscopy (AFM) techniques consist of a probe attached to a cantilever beam which is capable of indenting specific locations on a cell seeded on a substrate. Additionally, AFM techniques may be used to provide a high-resolution topographical map of the cell’s surface thus shedding light on the location of the nucleus and cytoskeletal elements which may be then compared to areas that have been indented. However, the AFM method does not permit for the entire cell to be indented which means that only localised characteristics are examined. Bader et al. (2002) used AFM to indent localised regions of the chondrocyte cytoplasm and found that the centre of the cell was the most resistance to deformation. The viscoelastic response of single chondrocytes isolated from various zones in articular
cartilage has been examined using AFM by Darling et al. (2006). It was found that chondrocytes from the superficial zone were significantly stiffer than cells from the middle/deep zone in terms of instantaneous and relaxed moduli and apparent viscosity. AFM has also been employed to characterise the mechanical properties of the chondron (Allen and Mao 2004; Ng et al. 2006).

2.3.5 Micropipette Aspiration

Micropipette aspiration is frequently used to investigate the biomechanical response of both adhered and non-adhered cells. Typically, cells are suspended in media or attached to a substrate, and a miniature pipette (5-15 μm in diameter) is used to apply a negative pressure on to the surface of the cell (Figure 2.13). The deformation of the cell is then captured using video technology and cell deformation may be calculated. Micropipette aspiration has been used to determine the mechanical properties of chondrocytes and its PCM (Guilak et al. 1999), and also the viscoelastic properties of healthy and osteoarthritic chondrocytes (Jones et al. 1999; Trickey et al. 2000; Trickey et al. 2006). Furthermore, disruption of the actin cytoskeleton by cytochalasin-D was found to alter the biomechanical response of chondrocyte during micropipette aspiration (Trickey et al. 2004). In contrast, disruption of the intermediate filaments and microtubules using acrylamide and colchicine did not have a significant effect on the mechanical properties of the cells.

2.4 Computational Modelling of Chondrocyte and Cartilage Mechanics

2.4.1 Chondrocyte Cell Models

Computational modelling has previously been used to simulate the response of cells to mechanical stimuli and to characterise the mechanical properties of the cell. Three types of continuum models, incorporating different representations of the cell material and with varying degrees of complexity, have commonly been used to predict chondrocyte deformation due to an applied load: (i) linear elastic model; (ii) viscoelastic model; and (iii) biphasic model.

Linear elastic models exhibit a linear relationship between stress and strain during deformation, and assume that the cell is incompressible, homogeneous, and isotropic. Linear elastic models have been used to determine the mechanical
properties (Young’s modulus and Poisson’s ratio) of chondrocytes during compression (Ofek et al. 2009a), in response to indentation (Shin and Athanasiou 1999; Ng et al. 2006), and of chondrosarcoma cells embedded in agarose gel during compression (Freeman et al. 1994).

Linear elasticity does not take into account the time-dependent behaviour of cells; hence, several approaches incorporating viscoelasticity have been developed. Viscoelastic models, such as the standard linear solid model, represent the cell material as a circuit consisting of springs and dashpots. The elastic component of the cells behaviour is represented as a spring, and a dashpot represents the viscous nature of the cell. The standard linear solid model can capture both the creep and stress relaxation behaviour of cells in response to an applied constant stress and step strain respectively. Material parameters such as instantaneous modulus, relaxed modulus, and apparent viscosity can be obtained when using viscoelastic models. Viscoelastic models have been employed to simulate the response of single chondrocytes to unconfined compression (Leipzig and Athanasiou 2005; Shieh and Athanasiou 2006), indentation (Koay et al. 2003), and micropipette aspiration (Guilak et al. 1999; Haider and Guilak 2000; Trickey et al. 2000; Baaijens et al. 2005; Trickey et al. 2006).

Biphasic models describe a material as having both a solid and fluid phase (Mow et al. 1980). When applied to cells, a biphasic model represents the interaction of both solid and fluid components within the cell membrane. The cytoskeleton, nucleus and organelles are depicted as solid components, creating a porous continuum for the fluid-like cytosol. Biphasic modelling can obtain the following mechanical properties of the cell: aggregate modulus, permeability and Poisson’s ratio. This model has been used to simulate the response of chondrocytes to compression (Bachrach et al. 1995; Wu et al. 1999; Guilak and Mow 2000) and micropipette aspiration (Trickey et al. 2006).

The aforementioned material models only represent the passive behaviour of the cell, and ignore the key mechanisms by which cells actively respond to mechanical stimuli. The inadequacy of passive viscoelastic cell models has been demonstrated by McGarry and McHugh (2008). It was shown that in order to replicate in vitro chondrocyte detachment, the stiffness of the cytoplasm must be
artificially increased as the cell spreads. A similar deficiency has been reported for the passive modelling of cells during parallel plate compression (McGarry 2009). These computational studies highlight the limited insight and predictive capability of passive material models.

As previously discussed, current experimental methods aim to investigate the underlying mechanisms involved in chondrocyte mechanotransduction. Hence, there is a need for a similar approach for computational cell models that can elucidate the key mechanisms by which cells actively respond to mechanical loads. An active modelling framework, which incorporates the biomechanisms underlying the formation, dissociation and contractility of the actin cytoskeleton, has recently been developed (Deshpande et al. 2006; Deshpande et al. 2007). This active formulation has been shown to accurately predict changes in cell contractility for various cell phenotypes seeded on micro-pillar arrays (McGarry et al. 2009), and cytoskeletal arrangements in cells attached to patterned substrates (Pathak et al. 2008). In addition, the model has also predicted the effect of cyclic substrate deformation on stress fibre orientations (Wei et al. 2008). A detailed description of this active bio-chemo-mechanical model is provided in Chapter 4. In this thesis, this active formulation is implemented to predict the role of the actin cytoskeleton in the response of chondrocytes to externally applied loading.

### 2.4.2 Cartilage Tissue Models

Numerous computational models based on finite element methods have been developed in order to investigate the mechanical behaviour of articular cartilage. Biphasic theory describes soft biological materials as a mixture of a solid matrix, hydrated with a fluid (Mow et al. 1980). When applied to articular cartilage, a biphasic model represents the interaction of the solid component (collagen fibres, proteoglycans, and chondrocytes) and fluid component (water and dissolved ions matrix) within the tissue. The total stress in the tissue is given by the sum of the solid and fluid stress. It is assumed that both the solid and fluid phases are present at every point in the tissue. The solid matrix is isotropic and linearly elastic, and both phases are assumed to be intrinsically incompressible. Biphasic models include the viscoelastic behaviour of soft biological materials by assuming that a drag force is generated when the inviscid fluid phase moves relative to the solid phase. However,
Chapter 2

biphasic models do not account for important features of cartilage such as anisotropy caused by collagen fibre arrangement and the swelling behaviour of the tissue. Biphasic models have previously been used to analyse numerous loading experiments involving articular cartilage tissue, such as unconfined compression (Armstrong et al. 1984; Guilak and Mow 2000), confined compression (Ateshian et al. 1997), indentation (Mak et al. 1987; Mow et al. 1989; Suh and Spilker 1994) and impact loading (Atkinson and Haut 1995; Garcia et al. 1998; Moo et al. 2012).

Articular cartilage is highly anisotropic due to the complex arrangement of collagen fibres throughout the depth of the tissue. Several computational models have been developed to account for the direction-dependent material behaviour of cartilage. Transverse isotropic models assume that all collagen fibres are aligned in the same direction, that is, an orthotropic material with one plane of isotropy (Wilson et al. 2005b). Compression (Cohen et al. 1998; Bursac et al. 1999) and impact loading (Garcia et al. 1998; Donzelli et al. 1999) have been simulated using transverse isotropic models.

A more complex approach taken to model the mechanical behaviour of cartilage has included the development of 2D fibre-reinforced material models (Li et al. 1999; Li and Herzog 2004; Wilson et al. 2005a; Wilson et al. 2006; García and Cortés 2007; Julkunen et al. 2007; Quinn and Morel 2007; Wilson et al. 2007). In a fibre-reinforced model, the tissue is represented as two components: (i) an isotropic gel matrix consisting of proteoglycans, chondrocytes, and water; (ii) a fibre network incorporating the anisotropic arrangement of type II collagen fibres. These models have been shown to be capable of simulating the creep and relaxation behaviour of mechanically loaded cartilage and have provided significant insight into the mechanical behaviour of cartilage in a 2D environment. Recently, fibre-reinforced constitutive models incorporating 3D collagen fibre configurations have been developed (Li et al. 2009; Pierce et al. 2009).

2.5 Summary and Conclusions

This section reviews the current literature regarding the structure and function of articular cartilage, chondrocytes, and, in particular, the actin cytoskeleton. Additionally, experimental techniques for investigating chondrocyte
mechanics are reviewed. Furthermore, computational models used to simulate the behaviour of chondrocytes and cartilage tissue in response to mechanical loading are presented.

Chondrocytes do not constitute the primary structural elements of articular cartilage. Yet, it is the response of the chondrocyte to external loading that regulates the production of the necessary proteins and molecules in order to maintain healthy tissue. The cytoskeleton of the chondrocyte is known to play a significant role in the response of chondrocytes to mechanical stimuli. The majority of the experimental studies demonstrate that the actin cytoskeleton is predominantly located cortically, composed of F-actin and associated proteins, and is distributed just beneath the plasma membrane. Despite the numerous computational models developed to simulate chondrocyte behaviour, the mechanisms by which chondrocytes actively respond to mechanical stimuli have been ignored. Single cell experimentation along with active computational models is a novel approach for investigating the role of the cytoskeleton in the response of cells to mechanical stimuli.
References


Shahin, K. and Doran, P. M. (2012). "Tissue engineering of cartilage using a mechanobioreactor exerting simultaneous mechanical shear and compression to simulate the rolling action of articular joints." *Biotechnology and Bioengineering*.


### Tables

Table 2.1 Computational models employed to simulate experimental chondrocyte deformation in response to external loading. The majority of the models listed below are based on the finite element method.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Material Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Cell Compression</td>
<td>Linear Elastic</td>
<td>(Ofek et al. 2009a)</td>
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<tr>
<td></td>
<td>Viscoelastic</td>
<td>(Koay et al. 2003)</td>
</tr>
<tr>
<td>Single Cell Shear</td>
<td>Linear Elastic</td>
<td>(Shin and Athanasiou 1999)</td>
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<td></td>
<td>Viscoelastic</td>
<td>(McGarry and McHugh 2008)</td>
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<td></td>
<td>Poroelastic</td>
<td>(Shin and Athanasiou 1999)</td>
</tr>
<tr>
<td>Micropipette Aspiration</td>
<td>Nonlinear elastic</td>
<td>(Baaijens et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>Viscoelastic</td>
<td>(Baaijens et al. 2005), (Trickey et al. 2006)</td>
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<tr>
<td></td>
<td>Biphasic</td>
<td>(Alexopoulos et al. 2005)</td>
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<tr>
<td></td>
<td>Poroelastic</td>
<td>(Baaijens et al. 2005), (Baaijens et al. 2005),</td>
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<td>Poroviscoelastic</td>
<td>(Baaijens et al. 2005), (Trickey et al. 2006)</td>
</tr>
<tr>
<td>In Situ Deformation</td>
<td>Biphasic</td>
<td>(Guilak and Mow 2000), (Kim et al. 2010), (Wu et al. 1999), (Han et al. 2007), (Wu and Herzog 2000)</td>
</tr>
</tbody>
</table>
Figure 2.1 Articular cartilage exhibits a structure that is suited to its biomechanical role of distributing loads and protecting surround bone. In the superficial zone, chondrocytes and collagen fibres aligned tangentially to the surface give the tissue high tensile strength and stiffness. The middle zone contains larger, more rounded chondrocytes and randomly aligned collagen fibres. Chondrocytes and collagen fibrils in the deep zone align perpendicular to the bone giving the tissue high compressive properties.
Figure 2.2 The structure of the cytoskeletal elements (a) actin microfilaments, (b) microtubules, (c) intermediate filaments. Reproduced from Blain (2009). **Figure blurred due to copyright reasons.**
Figure 2.3 The presence of ATP, Mg and K in the cytosol leads to the nucleation of G-actin monomers, which leads to the formation of long F-actin filaments. Adapted from Lodish et al. (2000). Figure blurred due to copyright reasons.
Figure 2.4 The cross-bridge cycling process of actin and myosin. The hydrolysis of ATP causes a single myosin head to pivot and exert traction on the attached actin filament. In each cross-bridge cycle, the myosin head moves about 5 nm towards the (+) end of the actin filament. Adapted from Alberts (2002). \textbf{Figure blurred due to copyright reasons.}
Figure 2.5 Immunocytochemistry images of intermediate filaments, actin filaments, and microtubules before (left) and after (right) treatment with acrylamide, cytochalasin-D and colchicine respectively. Reproduced from Ofek et al. (2009b). **Figure blurred due to copyright reasons.**
Figure 2.6 *In situ* actin cytoskeleton organisation in cartilage explants. (a) Single confocal microscope images of FITC conjugated phalloidin-labelled F-actin in chondrocytes from rat articular cartilage. Scale bars: 2 μm. (i) 3D reconstruction. (ii) Optical section at the cell periphery. (iii) Optical section in the mid-region of the cell. Reproduced from Durrant et al. (1999). (b) Single confocal microscope images of bovine cartilage sections labelled for actin filaments with TRITC phalloidin from different tissue zones: (i) tangential, (ii) middle; and (iii) deep. Reproduced from Langelier et al. (2000). *Figure blurred due to copyright reasons.*
Figure 2.7 Actin cytoskeleton organisation in chondrocytes cultured in a ‘3D environment’. (a) Confocal microscope z-stack slices of FITC phalloidin-labelled actin filaments from the centre (top) to the periphery (bottom) of a single chondrocyte (bovine) embedded in 4% agarose. Scale bar: 2 μm. Reproduced from Knight et al. (2001a). (b) Confocal microscope (i) z-stack slices of FITC phalloidin-labelled actin filaments from the centre (top) to the periphery (bottom) of a single chondrocyte (bovine) embedded in 4% agarose, and (ii) the corresponding 3D reconstruction. Scale bar: 5 μm. Reproduced from Knight et al. (2006b). (c) Single confocal microscope images illustrating non-organised (top), partially-organised (middle), and clearly-organised (bottom) FITC phalloidin-labelled actin filaments in single chondrocytes (bovine) embedded in 6% agarose. Scale bar: 2 μm. Reproduced from Idowu et al. (2000). Figure blurred due to copyright reasons.
Figure 2.8 (a) Single confocal microscope slice of Alexa 488 phalloidin-labelled F-actin in a human chondrocyte suspended in 1.2% alginate beads. Scale bar: 5 μm. Reproduced from Trickey et al. (2004). (b) Single confocal microscope slice of GFP-actin in a bovine primary chondrocyte suspended in media. Scale bar: 10 μm. Reproduced from Pravincumar et al. (2012) (c) Confocal section images of Alexa 564 phalloidin-labelled F-actin (red) in a chondrocyte cultured for 24 hours in 1% and 3% agarose gel. Scale bar: 10 μm. Reproduced from Chen et al. (2012). (d) Confocal slice of Alexa 564 phalloidin-labelled F-actin (red) in a chondrocyte embedded in 2% alginate gel. Scale bar: 5 μm. Reproduced from Haudenschild et al. (2011). **Figure blurred due to copyright reasons.**
Figure 2.9 *In vitro* actin cytoskeleton organisation in single bovine chondrocytes adhered to glass substrates for three hours. F-actin (red) is stained with: (a) AlexaFluor 647 phalloidin; and (b) rhodamine phalloidin. Image slices were taken using a fluorescent microscope. Cells in (a) and (b) are not to scale. Reproduced from (a) Ofek et al. (2009b) and (b) Leipzig et al. (2006). Figure blurred due to copyright reasons.
Figure 2.10 *In vitro* chondrocytes in monolayer displaying highly aligned stress fibre bundles. (a) Fluorescent microscope image slice of rabbit chondrocytes labelled with rhodamine phalloidin. Reproduced from Brown and Benya (1988). (b) Confocal microscope image of a human chondrocyte labelled with phalloidin. Scale bar: 10 μm. Reproduced from Haudenschild et al. (2009). (c) Confocal microscope image slice of a mouse chondrocyte labelled with rhodamine phalloidin (red). Scale bar: 10 μm. Reproduced from Woods et al. (2005). (d) Confocal microscope image slice of a bovine chondrocyte labelled with TRITC-conjugated phalloidin. Reproduced from Campbell et al. (2007). (e) Confocal microscope image slice of bovine chondrocytes
labelled with Alexa-phalloidin Reproduced from Chen et al. (2012). **Figure blurred due to copyright reasons.**

Figure 2.11 Single cell testing techniques: (a) whole-cell compression; (b) compression in agarose gel; (c) shear deformation; (d) AFM / nanoindentation; (e) micropipette aspiration.
Figure 2.12 Strain-controlled compression of single chondrocytes experiencing low strain (a-c) and high strain (d-f). Reproduced from Ofek et al. (2009b). Figure blurred due to copyright reasons.

Figure 2.13 (a) Brightfield and (b) confocal microscopy images of single chondrocytes during micropipette aspiration at increasing water pressures. Cells are transfected with eGFP actin. Reproduced from Bader and Knight (2008). Figure blurred due to copyright reasons.
3 Experimental Methods

3.1 Overview

The following chapter describes the experimental methods performed in this thesis. Articular cartilage was harvested from bovine tissue and single chondrocytes were isolated from the middle/deep zones of the tissue. Shear deformation was applied to individual cells by a tungsten probe. In order to monitor the deformed cell geometry, a video camera was used and individual frames were analysed using image analysis software. Immunocytochemistry techniques were used to observe alterations in the actin cytoskeleton, focal adhesions and the nucleus during applied shear. An additional series of shear experiments was carried out on cells where one of the three following cytoskeletal components was disrupted: actin filaments, intermediate filaments, and microtubules. All tissue dissections and experiments were conducted in the Musculoskeletal Bioengineering Laboratory of Prof. K.A. Athanasiou in Rice University, Texas, USA.

3.2 Articular Cartilage Harvest

The lower legs of mature steers, severed at the carpal joint, were obtained from a local abattoir (Doreck and Sons Packing Company, Santa Fe, Texas, USA) directly after slaughter. The samples were placed into a sealed container surrounded by ice and transported to the laboratory within 1 hour for harvesting of articular cartilage tissue. Under aseptic conditions, the skin and hoof were removed from the leg. The leg was then carefully washed using iodine solution and rinsed with deionised water. Tendons, ligaments and any remaining tissue were dissected until the fetlock joint was exposed (Figure 3.1). In order to obtain cartilage tissue from the condyle surface of the fetlock joint, zonal abrasion was performed, based on the method used by Darling et al. (2004). Firstly, a scalpel blade was firmly drawn across the surface in order to remove the top 10-20% of the tissue. This technique results in the removal of the superficial zone of the tissue. Tissue was then carefully sliced from the middle/deep zone and placed in a petri dish containing a solution of 0.2% collagenase type 2 and 0.3% dispase (Worthington Biochemical, Lakewood, NJ, USA).
3.3 Cell Isolation and Seeding

Single chondrocytes were isolated through overnight digestion at 37°C and 10% CO₂. After tissue digestion, the cell suspension was centrifuged and resuspended in DMEM media containing 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids (NEAA), 100 U/ml penicillin/streptomycin, and 0.25 μg/ml fungizone, at a concentration of 2x10⁵ cells/ml. DMEM media and supplements were purchased from Invitrogen, Carlsbad, CA, USA. Cut pieces (∼5 × 20mm) of untreated Superfrost® Plus glass microscopy slides (VWR, West Chester, PA, USA) were placed in a single well of a 6-well plate and covered with 3 ml of the cell suspension, yielding a cell seeding density of approximately 1x10⁶ cells/cm². Untreated glass slides were chosen for this experiment based on prior results which demonstrated that chondrocytes adhered well to this substrate, with a minimal baseline expression of focal adhesions and the actin cytoskeleton (Athanasiou et al. 1999). The 6-well plates were incubated at 37°C and 10% CO₂ for 3 hours. The 3 hour seeding time was determined in prior studies to be sufficient for sufficient chondrocyte attachment, as well as closely maintaining the cell’s in vivo morphology (Leipzig et al. 2006; Shieh and Athanasiou 2006). After 3 hours, the glass substrate containing the cells was removed from the incubator and placed in a petri dish on an IMT-2 inverted microscope (Olympus America, Melville, NY, USA). Supplemented DMEM media with 30 mM HEPES, pre-heated to 37°C, was added to the dish throughout the duration of the experiment. The HEPES buffer was used to prevent pH changes in the media as experiments were performed in ambient conditions.

3.4 Shear Deformation and Video Analysis

Shearing of single chondrocytes was performed using a modified cytodetachment apparatus (Athanasiou et al. 1999; Hoben et al. 2002; Huang et al. 2003). This device was adapted from its original setup to allow for the video capture of cells experiencing mechanical deformation (Athanasiou et al. 1999; Hoben et al. 2002; Huang et al. 2003). A shear deformation was applied to individual cells by a 50.8 μm diameter tungsten probe (Advanced Probing Systems, Inc., Boulder, CO, USA). Initially, the probe was positioned adjacent to the cell, 4 μm above the substrate (Figure 3.2 (a)). Control over the distance between the bottom of the probe and the glass substrate was achieved via linear x-y-z micrometers, as confirmed by
visualisation on the microscope. The height of the probe from the glass substrate (4 µm) was kept constant as it was hypothesised that the highly anisotropic nature of the cytoplasm would be affected by probe height, and as a result the response of the cell to deformation would be probe height dependent. The fixed (top) end of the probe was then moved horizontally towards the cell at a constant speed of 4µm/s using a piezoelectric motor. The free (bottom) end of the probe indents the cell, leading to a shear deformation (Figure 3.2 (b)). Due to the resistance of the cell to deformation, a deflection is imparted on the free end of the probe relative to the known position of the fixed end. Using cantilever beam theory, this deflection (δ) is used to determine the force required to deform the cell throughout the shear experiment:

\[ F = \frac{3EI}{L^3} \delta \]  

where \( E \) (Young’s modulus), \( I \) (area moment of inertia), and \( L \) (cantilever beam length) are known parameters of the tungsten probe. The Young’s modulus, area moment of inertia and length of the probe are 394.5 GPa, 3.27 x 10^{-19} m^4, and 17.4 mm respectively. Cell deformation was monitored using an AVC-D7 CCD camera (Sony USA). Videos (Figure 3.3) were saved as an AVI file at 640 × 480 resolution for analysis. Individual frames were extracted and analysed using Videomach 4.2 (Gromada.com) and Microsoft Paint 5.1 (Microsoft Corporation, Redmond, WA, USA) respectively. A pixel-to-micron ratio of 7.0 was employed in all image subsequent analysis. Images of the initial cell geometry, initial cell-probe contact, and the shear event every 0.25 seconds thereafter were examined. The deflection of the cantilever (δ) was calculated by comparing the true displacement of the probe (via video analysis) with the prescribed piezoelectric displacement for each time step. Probe indentation is defined as the forward-most position of the probe minus the back edge position of the cell.

3.5 Fluorescence Microscopy

To observe alterations in the actin cytoskeleton and focal adhesions, cells were fixed at maximum shear deformation and stained using fluorescent antibodies. Chondrocytes were seeded using the protocol described above, except on to
microscope slides that were etched with an indelible marker along their underside. After 3 hours of culture, a digital image was taken of the cell seeding pattern in one of the etched regions on the slide, in order to identify specific chondrocyte locations (Figure 3.4). The slides were then placed flat inside a petri dish on the IMT-2 microscope such that the cells could be viewed bottom-up. The same region of the slide that was previously imaged digitally was located on the microscope and a single chondrocyte within this region was selected for shearing and fixation. The probe was placed immediately adjacent to this cell and translated horizontally toward the cell, similar to the protocol described above. The cell was held at the deformed position for 10 minutes, at which point 4% paraformaldehyde was carefully added to the dish and fixation was allowed to occur for an additional 10 minutes. After the cell was fixed, the glass substrate was removed from the petri dish, washed three times with PBS, blocked with 10% FBS, and then permeabilised with 0.1% Triton X-100 in PBS for cell staining. Cells were then incubated with AlexaFluor 647 Phalloidin (Invitrogen, Carlsbad, CA, USA) for actin visualisation, mouse anti-Vinculin primary antibody (Sigma-Aldrich, St. Louis, MO, USA) followed by a goat anti-mouse secondary anti-body (AlexaFluor 488; Invitrogen, Carlsbad, CA, USA) for focal adhesion imaging, and Hoescht’s dye (Invitrogen, Carlsbad, CA, USA) for nuclei staining. The cells were viewed with an Axioplan 2 microscope (Carl Zeiss, Oberbrocken, Germany) using a 100× oil immersion objective. The nucleus, actin filaments, and focal adhesion were imaged using filters that were excited at wavelengths of 359, 493, and 540 nm respectively; with emitted fluorescence appearing at 461, 517, 565 nm respectively. Exposure times were kept consistent for each cell and were 3 ms for the nucleus, 250 ms for focal adhesions, and 567 ms for the actin cytoskeleton. Z-stacks of control and sheared cells were acquired at 0.2 μm intervals using the Metamorph 4.15 software package (Universal Imaging Corp., Downingtown, PA).

### 3.6 Cytoskeletal Disrupting Treatment

An additional series of shear experiments were performed where one of the three cytoskeletal components were disrupted by adding a specific cytoskeletal disrupting agent to the cell suspension during cell seeding, and to the supplemented media used during testing. The disruption of the actin cytoskeleton, intermediate
filaments, or microtubules was carried out by the addition of cytochalasin-D (cyto-D, 2 µM), acrylamide (40 mM), or colchicine (10 µM), respectively. All cytoskeletal disrupting agents were obtained from Sigma Aldrich (St. Louis, MO, USA). The concentrations for the specific cytoskeletal disrupting agents were chosen based upon previous studies demonstrating their effectiveness with chondrocytes (Madsen et al. 1979; Takigawa et al. 1984; Guilak et al. 1999; Trickey et al. 2004; Ofek et al. 2009).

### 3.7 Actin Intensity

The re-organisation of the actin cytoskeleton following shear was analysed in cells which were fluorescently stained (n = 5). Specifically, the fluorescence intensity of actin was determined using Image J software (rsb.info.nih.gov). Firstly, an image slice was chosen at a focal plane near the base of the cell. A region of interest was selected around the edge of a cell and the average intensity (average gray value (AGV)) was measured. AGV is defined as the total of all gray values divided by the total number of pixels. To obtain a reading for averaged background fluorescence, a region nearby the cell was selected and the average intensity was measured. The cell’s relative intensity was then defined as the difference between the average intensity of the cell and the average intensity of the background.

### 3.8 Data Analysis

All statistical analysis was performed using Microsoft Excel (Microsoft Corp., Dublin, Ireland) and Minitab ver. 15.1 (Minitab Ltd., Coventry, UK). A single factor ANOVA was used with a Fisher’s Least Significant Difference post-hoc test to test the effects of the cytoskeletal disrupting agents on the force response of cells during shear and to identify differences between the fluorescent intensity of cellular zones. Significance was defined as p < 0.05 throughout the study.
References


Figures

Figure 3.1 Photograph of the fetlock joint from a mature steer. Articular cartilage was harvested from this joint for the experimental studies conducted in this thesis.
Figure 3.2 Schematic image of cell and probe: (a) prior to indentation; (b) during shear indentation. (Note: probe and deflection $\delta$ are not to scale in (a) and (b)).

Figure 3.3 Experimental image of cell and probe: (a) prior to indentation; (b-c) during shear indentation. The initial geometry of the cell and probe deflection were determined from individual frames. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Figure 3.4 Photograph of adhered chondrocytes on glass substrates used to identify chondrocyte locations prior to applied shear. The dashed lines represent a region that was etched with a permanent marker.
4 Theory

4.1 Overview

In this chapter, the theoretical basis for the finite element modelling performed in Chapters 5, 6 and 7 is outlined. Firstly, an overview of continuum mechanics and large/finite deformation kinematics is provided in section 4.2. In order to model the mechanical behaviour of cartilage tissue, the constitutive laws for hyperelasticity (section 4.2.4) and anisotropic hyperelasticity (section 4.2.5) are outlined. A theoretical framework for stress fibre remodelling and contractility is presented in section 4.3. This framework is used to simulate the behaviour of the chondrocyte actin cytoskeleton in this thesis. Finally, a synopsis of the finite element solver used in this work, Abaqus Standard, is given in section 4.4.

4.2 Continuum Mechanics

4.2.1 Introduction

The following section presents a brief overview of continuum mechanics. A more detailed discussion of continuum mechanics may be found in Athanasiou and Natoli (2008). For the purposes of clarity, vectors, tensors and matrices are denoted with a bold type face. Capital letters are used for tensors and matrices, and small letters are given for vectors. Index notation can be employed in order to simplify the representation of vector equations. For example, the dot product of two vectors (e.g. displacement and velocity) in 3D can be written using summation convention as the following:

\[ \mathbf{v} \cdot \mathbf{u} = v_1 u_1 + v_2 u_2 + v_3 u_3 = \sum_{i=1}^{3} v_i u_i \]  (4.1)

Second order tensors, such as stress and strain, have nine components in 3D. For example, the component \( A_{ij} \) of a second order tensor \( \mathbf{A} \), has subscripts that define its location within the tensor (i.e. \( i \) and \( j \)). Fourth order tensors describe linear tensor functions of second order tensors. For example, the second order tensor of stress, \( \mathbf{\sigma} \), is related to the second order tensor of strain, \( \mathbf{\varepsilon} \), through the fourth order elastic modulus tensor, \( \mathbf{C} \), as follows:
The concept of a continuum allows properties such as density, displacement, temperature, etc. to be represented as continuous (in a mathematical sense) functions of position and time.

### 4.2.2 Deformation and Strain

Large/finite deformation kinematics can be described by considering the deformation of a body with respect to an initial configuration of the same body. As shown in Figure 4.1, initially an undeformed body $V$ is in the reference configuration. Deformation leads to $V^*$ in the current configuration. The deformation of a particle on the body is defined by considering the position vector $x$ on $V$, and the position vector $y$ on $V^*$. The displacement vector of the particle is given as:

$$ u(x) = y(x) - x \quad (4.3) $$

and the velocity vector can be obtained by differentiating the displacement vector with respect to time $t$:

$$ \frac{du}{dt} = \dot{u} = v(x) \quad (4.4) $$

The deformation gradient tensor, $F$, gives the change in position of a particle relative to its initial point. As shown in Figure 4.1, the mapping of an infinitesimal material fibre in the reference configuration, $dx$, to that fibre in the current configuration, $dy$, can be expressed as:

$$ dy = F \cdot dx \Rightarrow F = \frac{\partial y}{\partial x} \quad (4.5) $$

From the above expression, the velocity gradient, $\hat{L}$, can be derived and is given as:

$$ \hat{L} = \frac{\partial v}{\partial x} = \dot{F} \quad (4.6) $$

and the spatial velocity gradient is:

$$ L = \frac{\partial v}{\partial y} = \dot{F} \cdot F^{-1} \quad (4.7) $$

The determinant of the deformation gradient, $F$, is known as the Jacobian of the deformation, $J$, such that:
\[ J = \text{det}(F) \]  
(4.8)

The deformation gradient, \( F \), is a second-order tensor (\( \text{det}(F) > 0 \)), and can be decomposed such that:

\[ F_{ij} = R_{ik}U_{kj} = V_{ik}U_{kj} \]  
(4.9)

Where \( U \) and \( V \) are symmetric tensors and \( R \) is orthogonal. Thus, the deformation can be considered as a rotation \( (R) \) followed by a stretch \( (U, \text{the right stretch tensor}) \), or a stretch \( (V, \text{the left stretch tensor}) \) followed by a rotation \( (R) \). The right Cauchy-Green deformation tensor, \( C \), can be defined as:

\[ C = F^TF \]  
(4.10)

and the left Cauchy-Green deformation tensor, \( B \), as:

\[ B = FF^T \]  
(4.11)

The Green or Lagrangian strain tensor, \( E \), is defined as:

\[ E = \frac{1}{2}(F^TF - I) \]  
(4.12)

where \( I \) is the identity tensor. Strain rate is given as:

\[ \dot{E} = \frac{1}{2}(F^T \cdot F + F^T \cdot F) \]  
(4.13)

The symmetric rate of deformation tensor \( D \) is defined as:

\[ D = \text{sym}(L) = \frac{1}{2}(L^T + L) \]  
(4.14)

The logarithmic strain tensor, \( \varepsilon \), is obtained by integrating the rate of deformation tensor \( D \) with respect to time:

\[ \varepsilon(t) = \int_0^t D \, dt \]  
(4.15)

4.2.3 Stresses

The Cauchy (true) stress tensor, \( \sigma \), can be defined as the force per unit area on the current configuration. It is related to the traction, \( t \), on an internal or external surface and the unit normal vector to the surface, \( n \), as shown by:

\[ t = \sigma \cdot n \]

\[ t_i = \sigma_{ij}n_j \]  
(4.16)

This stress tensor is also symmetric (i.e. \( \sigma_{ij} = \sigma_{ji} \)). The principal stresses can be determined from the solution to the eigenvalue equation.
\((\sigma - \sigma I) \cdot n = 0\) \hspace{1cm} (4.17)

where \(\sigma\) is the principal stresses and \(I\) is the second order identity tensor. The characteristic equation of this problem can be expressed as:

\[
\sigma^3 - l_1 \sigma^2 - l_2 \sigma - l_3 = 0
\] \hspace{1cm} (4.18)

where \(l_1, l_2,\) and \(l_3\) are the first-order stress invariants, and are given as:

\[
l_1 = \sigma_{kk}
\] \hspace{1cm} (4.19)

\[
l_2 = \frac{1}{2} (\sigma_{ij} \sigma_{ij} - l_1^2)
\] \hspace{1cm} (4.20)

\[
l_3 = \det(\sigma)
\] \hspace{1cm} (4.21)

The three invariants can also be expressed in terms of the principal stresses:

\[
l_1 = \sigma_1 + \sigma_2 + \sigma_3
\] \hspace{1cm} (4.22)

\[
l_2 = -(\sigma_1 \sigma_2 + \sigma_2 \sigma_3 + \sigma_3 \sigma_1)
\] \hspace{1cm} (4.23)

\[
l_3 = \sigma_1 \sigma_2 \sigma_3
\] \hspace{1cm} (4.24)

Generally, stress can be split into two parts, consisting of the hydrostatic (volume changing) and deviatoric (shape changing) stress fields. The mean hydrostatic pressure, \(p\), which is a scalar, is defined as:

\[p = -\frac{1}{3} I_1 = -\frac{\sigma_{kk}}{3}\] \hspace{1cm} (4.25)

The deviatoric stress, \(S\), is defined as:

\[S = \sigma - pI\] \hspace{1cm} (4.26)

The equivalent tensile stress (von Mises) is commonly used in this thesis and is defined as:

\[
\bar{\sigma} = \sigma_{mises} = \sqrt{3l_2(S)} = \sqrt{\frac{3}{2} S_{ij} S_{ij}}.
\] \hspace{1cm} (4.27)

4.2.4 Hyperelasticity

This section describes the constitutive behaviour of a hyperelastic material in the context of an isotropic response. Hyperelastic materials are described in terms of a ‘strain energy potential’, \(U(\varepsilon)\), which defines the strain energy stored in the material per unit of reference volume (volume in the initial configuration) as a function of the deformation at that point in the material. Hyperelastic material models are used in this thesis to simulate the behaviour of the PCM and in some
cases the ECM of cartilage tissue. Additionally, the chondrocyte nucleus and in some cases the cytoplasm are modelled as hyperelastic materials.

The neo-Hookean form of the strain-energy potential is used in this thesis. For isotropic material behaviour, the strain energy per unit of reference volume, $U$, can be expressed as:

$$U = C_{10}(\bar{I}_1 - 3) + \frac{1}{D_4}(J - 1)^2$$  \hfill (4.28)

where $C_{10}$ and $D_4$ are material parameters. $\bar{I}_1$ is the first deviatoric strain invariant, defined as:

$$\bar{I}_1 = \bar{\lambda}_1^2 + \bar{\lambda}_2^2 + \bar{\lambda}_3^2$$  \hfill (4.29)

where the deviatoric stretches, $\bar{\lambda}_i$, are defined as:

$$\bar{\lambda}_i = J^{-1/3}\lambda_i$$  \hfill (4.30)

where the Jacobian $J$ is the total volume ratio. $\lambda_i$ are the principal stretches ($\lambda_1, \lambda_2, \lambda_3$), and are defined as the eigenvalues of the symmetric right Cauchy stretch tensor. Thermal volume strain is not considered. $C_{10}$ and $D_4$ are related to the shear modulus ($\mu$), elastic modulus ($E$), bulk modulus ($K$), and Poisson’s ratio ($\nu$) as follows:

$$\frac{E}{2(1 + \nu)} = \mu = 2C_{10}$$  \hfill (4.31); \quad $$\frac{E}{3(1 - 2\nu)} = K = \frac{2}{D_4}$$  \hfill (4.32)

4.2.5 Anisotropic Hyperelasticity

Under large deformations, cartilage exhibits highly anisotropic and non-linear elastic behaviour due to the re-arrangement of type II collagen fibres within the proteoglycan/water gel matrix of the tissue. As stated in the previous section, hyperelastic materials are described in terms of a ‘strain energy potential’. In this thesis, a strain-based formulation is used to represent the strain energy potential of anisotropic hyperelastic materials. In this case, the strain energy potential can be expressed directly in terms of the components of the Green strain tensor ($\varepsilon^G$), such that:

$$U = U(\varepsilon^G)$$  \hfill (4.33)

where $\varepsilon^G = \frac{1}{2}(C - I)$ is Green’s strain; $C = F^T F$ is the right Cauchy-Green strain tensor; $F$ is the deformation gradient; and $I$ is the identity matrix. Without loss of generality, the strain energy function can be expressed in the form:
where $\varepsilon^G = \frac{1}{2}(\bar{C} - I)$ is the modified Green’s strain; $\bar{C} = J^{-\frac{2}{3}}C$ is the distortional part of the right Cauchy-Green strain; and $J$ is the volume change.

In this thesis, the form of the strain energy potential used is based on that proposed by Holzapfel et al. (2000) and Gasser et al. (2006) for modelling arterial layers with distributed collagen fibre orientations. The anisotropic material behaviour of the ECM, incorporating collagen fibre orientation, is modelled using the Holzapfel-Gasser-Ogden strain energy potential in Abaqus (2012) where:

$$U = C_{10}(\bar{I}_1 - 3) + \frac{1}{D}\left(\frac{(J)^2 - 1}{2} - \ln J\right)$$

$$+ \frac{k_1}{2k_2} \sum_{\alpha=1}^{N} \{ \exp[k_2(\bar{E}_\alpha)^2] - 1 \}$$

with

$$\bar{E}_\alpha \equiv \kappa(\bar{I}_1 - 3) + (1 - 3\kappa)(\bar{I}_{4(\alpha\alpha)} - 1)$$

where $U$ is the strain energy per unit of reference volume; $C_{10}, D, k_1, k_2$ and $\kappa$ are material parameters; $N$ is the number of families of fibres ($N \leq 3$); $\bar{I}_1$ is the first invariant of $\bar{C}$, $\bar{I}_{4(\alpha\alpha)}$ are pseudo-invariants of $\bar{C}$ and $A_\alpha$, and may be defined as:

$$\bar{I}_{4(\alpha\alpha)} = A_\alpha \cdot \bar{C} \cdot A_\alpha$$

where $A_\alpha, (\alpha = 1, ..., N)$, is a set of unit vectors that characterises the direction of fibres in the reference configuration.

The model assumes that the directions of the collagen fibres within each family are dispersed about a mean preferred direction. The parameter $\kappa \ (0 \leq \kappa \leq 1/3)$ describes the dispersion of the fibres. If $\rho(\Theta)$ is the orientation density function that characterises the distribution (it represents the normalised number of fibres with orientations in the range $[\Theta, \Theta + d\Theta]$ with respect to the mean direction), the parameter $\kappa$ is defined as

$$\kappa = \frac{1}{4} \int_0^\pi \rho(\Theta) \sin^3 \Theta d\Theta$$
It is also assumed that all families of fibres have the same mechanical properties and the same dispersion. The fibres are perfectly aligned when \( \kappa = 0 \). When \( \kappa = 1/3 \), the fibres are randomly distributed and the material becomes isotropic.

The strain-like quantity \( \tilde{E}_a \) characterises the deformation of fibres with \( \tilde{E}_a = \tilde{I}_{\kappa(aa)} - 1 \) for perfectly aligned fibres \( (\kappa = 0) \), and \( \tilde{E}_a = (\tilde{I}_1 - 3)/3 \) for randomly distributed fibres \( (\kappa = 1/3) \).

The first two terms in the strain energy function represent the contributions of the isotropic matrix material, and the third term represents the contributions of the collagen fibres. The model assumes that collagen fibres can support only tension, and that they buckle under compressive loading.

### 4.3 Theoretical Framework for Active Stress Fibre Remodelling and Contractility

The actin cytoskeleton provides the active machinery by which cells generate forces. It consists of stress fibres, which are comprised of actin filaments and the motor protein myosin. Contractile forces are generated via a cross-bridge cycling process, similar to that observed for skeletal muscle, whereby the hydrolysis of ATP causes the myosin head to pivot and exert traction on the attached actin filament. Regarding the remodelling of the actin cytoskeleton, two key observations should be noted: (i) stress fibres assemble due to a signalling cascade (such as RhoA/Ca\(^{2+}\)) in the cell (Burridge and Chrzanowska-Wodnicka 1996; Alberts et al. 2002); and (ii) stress fibres dissociate due to a reduction in tension in the cell cytoplasm (Franke et al. 1984; Burridge and Chrzanowska-Wodnicka 1996). From these two observations, a first order kinetic equation has been proposed (Deshpande et al. 2006) that describes the remodelling of the actin cytoskeleton:

\[
\frac{d\eta}{dt} = [1 - \eta] \frac{Ck_f}{\theta} - \left[1 - \frac{T}{T_0}\right] \eta k_b \frac{\theta}{\theta} \quad (4.39)
\]

where \( \eta \), \( (0 \leq \eta \leq 1) \), is the non-dimensional stress fibre activation level. The first term on the right hand side describes the rate of formation of stress fibres in response to a signal \( C \). The second term on the right hand side describes the rate of dissociation of stress fibres when the tension in the fibre drops below the isometric
tension $T_o$. $\theta$ is a constant that controls the decay rate of an activation signal. $k_f$ and $k_b$ are reaction rate constants.

The complete model of contractility and remodelling of stress fibres is depicted in Figure 4.2 (a-d). As shown in Figure 4.2 (a), an exponentially decaying signal $C = \exp(-t/\theta)$ (as observed for chondrocytes (Roberts et al. 2001)) leads to the formation of a contractile stress fibre bundle. If the stress fibre is fully activated ($\eta = 1$), it will produce a maximum isometric tension $T_{max}$. If the stress fibre shortens, due to its own active contractility or due to an externally applied load, its tension-velocity relationship is based on the classical cross-bridge cycling model developed for actin-myosin interactions in skeletal muscle (Hill 1938), whereby the stress fibre produces lower tension when the shortening velocity is large (Figure 4.2 (b)). When the fibre lengthens, for example upon the application of an external load, it yields, producing a tension equal to the isometric tension for all lengthening velocities. Finally, as shown in Figure 4.2 (c), when the stress fibre shortens, the associated reduction in tension (Eqn. 4.24) leads to dissociation of the stress fibre, thus lowering the activation level $\eta$. This in turn lowers the stress fibre contractility with the reduced isometric tension given as $T_o = \eta T_{max}$, as illustrated by the altered tension velocity relationship (blue dashed curve, Figure 4.2 (b)). The Hill-like (Hill 1938) equation used to model the contractility of stress fibres is specified as:

$$\frac{T}{T_0} = \begin{cases} 
0 & \frac{\dot{\epsilon}}{\dot{\epsilon}_0} < -\frac{\eta}{\tilde{k}_v} \\
1 + \frac{\tilde{k}_v}{\eta} \left(\frac{\dot{\epsilon}}{\dot{\epsilon}_0}\right) - \frac{\eta}{\tilde{k}_v} \leq \frac{\dot{\epsilon}}{\dot{\epsilon}_0} \leq 0 \\
1 & \frac{\dot{\epsilon}}{\dot{\epsilon}_0} > 0
\end{cases} \quad (4.40)$$

where $\dot{\epsilon}$ is the fibre contraction / extension strain rate, and the Hill-type constant $\tilde{k}_v$ is the fractional reduction in fibre stress upon an increase in shortening strain rate of $\dot{\epsilon}_0$.

This active stress fibre formulation (Deshpande et al. 2006) (Figure 4.2 (a-c)) is implemented into a 3D framework (Ronan et al. 2012). The stress fibre contractility model is implemented in a finite element code (Abaqus Ver. 6.9 (Simulia, RI, USA)) via a user-defined material subroutine. It is important to note
that the remodelling and contractility of the actin cytoskeleton is simulated throughout the entire cell cytoplasm, providing a fully predictive active model. At every integration point in the finite element mesh the theoretical framework for stress fibre remodelling and contractility is solved in 240 evenly spaced directions in 3D space. To illustrate this important point, the upper insert in Figure 4.2 (d) depicts a point in the cell where an even distribution of stress fibres is computed in all directions. In contrast, the lower insert depicts a point in the cell where a highly activated stress fibre is computed in one direction, with stress fibres dissociating in other directions, giving a more aligned fibrous distribution. This simple illustration demonstrates that simulations allow the actin cytoskeleton to actively evolve throughout the entire cell cytoplasm, depending on the signal strength and stress state in the cytoplasm. Hence a full prediction of the distribution of an inhomogeneous anisotropic contractile actin cytoskeleton is provided by this active 3D framework. By integrating the stress over each fibre direction, the active stress tensor is determined.

In order to implement the active stress fibre formulation in 3D, a representative volume element (RVE) is used (Deshpande et al. 2007). The RVE is defined as a sphere with radius $\rho$ containing stress fibres that are equally distributed in 3D space such that the distance between each fibre is minimised (Ronan et al. 2012). The orientation of any fibre within the RVE is defined using the unit vector:

$$ m = \sin(\omega) \cos(\phi) x_1 + \sin(\omega) \sin(\phi) x_2 + \cos(\omega) x_3 $$  \hspace{1cm} (4.41)

where $x_i$ are the unit base vectors for a Cartesian basis in the current configuration, $\omega$ is the angle of the fibre with respect to the $x_3$-axis, and $\phi$ is the angle that the projection of the fibre in the $x_{12}$ plane makes with the $x_1$-axis.

The stress in the material is defined using the Cauchy stress, and rigid body fibre rotations can be determined from the deformation gradient $F$ by:

$$ R_{ij} = F_{im} U_{mj}^{-1} $$  \hspace{1cm} (4.42)

where $R$ is the rotation tensor, and $U$ is the stretch tensor. The strain rate ($\dot{\varepsilon}_f$) in an arbitrary direction $m$ can be determined from the strain rate in the RVE using the following equation:
The contribution of all the fibres to the stress state in the RVE can be calculated by integrating over the volume:

\[ \sigma_{ij} = \frac{1}{V} \int \sigma_f(\omega, \varphi) m_i m_j \, dV \]  

(4.44)

This integral can be approximated numerically by considering a finite number of fibres that extend from the centre of the RVE to equidistant points on the surface of the sphere. The stress tensor in the Cartesian basis generated by the active stress fibre contractility is given as:

\[ \sigma_{ij}^{ACTIVE} = \sum_{k=1}^{n} \frac{\sigma_f(\omega_k, \varphi_k)}{n} m_i m_j(\omega_k, \varphi_k) \]  

where \( n \) is the number of fibre orientations.

This active framework is placed in parallel with a neo-Hookean hyperelastic formulation (Ronan et al. 2012), which represents the passive non-contractile cytoplasm material \( E_{cyto} \) surrounding the actin cytoskeleton. The passive stress tensor is given as:

\[ \sigma_{ij}^{PASSIVE} = \frac{2}{J} C_{10} \left( \bar{B}_{ij} - \frac{1}{3} \bar{B}_{kk} \delta_{ij} \right) + \frac{2}{D_1} (J - 1) \]  

(4.46)

where the deviatoric left Cauchy-Green tensor \( \bar{B} \) is determined from the deformation gradient \( F \):

\[ \bar{B}_{ij} = \frac{B_{ij}}{J^{2/3}} = \frac{F_{ik} F_{jk}}{(\epsilon_{lmn} F_{1l} F_{2m} F_{3n})^{2/3}} \]  

(4.47)

where \( \epsilon_{lmn} \) is the permutation tensor; and \( \epsilon_{lmn} = 1 \) if \( (l, m, n) \) is an even permutation of \((1, 2, 3)\), -1 if it is an odd permutation, and 0 if any index is repeated.

The material constants for the neo-Hookean model are given, from Eqns. 4.31 and 4.32, in terms of the Young’s Modulus \( E \) and Poisson’s ratio \( \nu \) as \( C_{10} = E/(1 + \nu) \) and \( D_1 = 6(1-2\nu)/E \).
The components of the total stress tensor at each integration point in the cytoplasm (Figure 4.2 (e)) are given as:

\[
\sigma_{ij}^{\text{TOTAL}} = \sigma_{ij}^{\text{ACTIVE}} + \sigma_{ij}^{\text{PASSIVE}}
\]  

(4.48)

In this thesis, the term cytoplasm refers to the cell body excluding the nucleus. In accordance with Eqn. 4.48, the cytoplasm consists of actively contractile stress fibres (which make up the actin cytoskeleton) in parallel with a passive non-contractile material (passive cytoplasm), which can be taken to represent microtubules, intermediate filaments, organelles, the cytosol etc.

In order to show the average distribution of actin, the average stress fibre intensity level (\(\bar{\eta}\)) is plotted at every point in the cytoplasm:

\[
\bar{\eta} \equiv \frac{1}{n} \sum_{k=1}^{n} \eta_k
\]  

(4.49)

where \(\eta_k\) (0 ≤ \(\eta\) ≤ 1) is the dimensionless stress fibre activation level; and \(n\) is the total number of discrete fibre orientations at each point. A sensitivity analysis of the number of fibre directions \(n\) demonstrated that convergence is achieved for \(n = 240\) (Ronan et al. 2012).

The circular variance measures the tendency of stress fibres to bundle in a dominant direction:

\[
\Pi = \eta_{\text{max}} - \bar{\eta}
\]  

(4.50)

where \(\eta_{\text{max}}\) is the maximum polymerisation level. \(\Pi\) can vary from 0 to 1. Low values of \(\Pi\) indicate that the actin cytoskeleton is not highly aligned in a dominant direction, but rather is smeared in appearance. On the other hand, a high value of \(\Pi\) indicates that stress fibres are highly aligned in specific dominant directions. Contour plots of the circular variance have been shown to correspond to fluorescent or confocal images in which background labelled actin has been removed (Pathak et al. 2008; McGarry et al. 2009).
4.4 Abaqus Standard

The word “implicit” in the context of Abaqus Standard refers to the method by which the state of a finite element model is updated from time $t$ to $t + \Delta t$. A fully implicit procedure means that the state at $t + \Delta t$ is determined based on information at time $t + \Delta t$, meaning iteration and convergence is required for solution. This is commonly implemented using a form of the Newton-Raphson (N-R) method, as presented below. For illustrative purposes and for simplicity the small strain case is presented and matrix/vector notation is used. The principle of virtual work is a fundamental equation upon which this method is based:

$$\int_V \delta \varepsilon^T \sigma dV = \int_S \delta u^T t dS \quad (4.51)$$

where the equilibrium is enforced on a reference volume, $V$, which is bounded by a surface, $S$. $\sigma$ and $t$ are the stress and surface traction vectors respectively, while $\delta \varepsilon$ and $\delta u$ are the virtual strain and virtual displacement vectors. The integrals of Eqn. (4.51) are taken over a finite element mesh with individual elements “$e$” of volume $V_e$ and surface $S_e$, and are evaluated using:

$$\delta \varepsilon = B_e \delta u_e \quad (4.52)$$

and

$$\delta u = N_e \delta u_e \quad (4.53)$$

where $N_e$ and $B_e$ are the element shape function and shape function gradient matrices respectively, and $\delta u_e$ are the nodal displacements. Substituting these into Eqn. (4.51) and rearranging, the following expression is generated:

$$\sum_e \int_{V_e} \delta u_e^T B_e^T \sigma (u_e) dV = \sum_e \int_{S_e} \delta u_e^T N_e^T t dS \quad (4.54)$$

where the summation is over all elements “$e$” in the finite element mesh. Note that the stress vector is dependent on $u_e$, the elemental vector of nodal displacements. Performing the summation, which in effect means assembling elemental quantities into global quantities, and eliminating the arbitrary virtual quantities yields the following global expression:

$$\int_V B^T \sigma(u) dV = \int_S N^T t dS \quad (4.55)$$
where $\mathbf{u}$ is the global nodal displacement vector for the mesh. A set of global equations in $\mathbf{u}$ for the out of balance force, $\mathbf{G}$, can then be assembled as follows:

$$
\mathbf{G}(\mathbf{u}) = \int_\Omega B^T \sigma(\mathbf{u}) dV - \int_\Sigma \mathbf{N}^T t dS = 0 \quad (4.56)
$$

In general, for non-linear problems involving non-linear constitutive laws and/or non-linear boundary conditions, Eqn. (4.56) is non-linear, and is usually solved by incremental methods, where loads/displacements are applied in time steps, $\Delta t$, up to an ultimate time, $t_{final}$.

Within each increment, the state of the analysis is updated iteratively using the N-R method from time $t$ to $t + \Delta t$ to solve for $\mathbf{u}^{t+\Delta t}$. An estimation of the roots of Eqn. (4.44) is made such that, for the $i^{th}$ iteration:

$$
\mathbf{d} \mathbf{u}_{i+1} = \mathbf{u}_{i+1}^{t+\Delta t} - \mathbf{u}_{i}^{t+\Delta t} = - \left[ \frac{\partial \mathbf{G}}{\partial \mathbf{u}} \right]^{-1} \mathbf{G}(\mathbf{u}_{i}^{t+\Delta t}) \quad (4.57)
$$

where $\mathbf{u}_{i}^{t+\Delta t}$ is the vector of nodal displacements for the $i^{th}$ iteration at time $t + \Delta t$ and $\mathbf{u}_{i+1}^{t+\Delta t}$ is an improved estimate of the nodal displacements relative to $\mathbf{u}_{i}^{t+\Delta t}$. The partial derivative on the right hand side of the equation is the Jacobian matrix of the governing equations and can be referred to as the global stiffness matrix, $\mathbf{K}_G$. Eqn. (4.45) is manipulated and inverted to produce a system of linear equations:

$$
\mathbf{K}_G(\mathbf{u}_{i}^{t+\Delta t}) \delta \mathbf{u}_{i+1} = -\mathbf{G}(\mathbf{u}_{i}^{t+\Delta t}) \quad (4.58)
$$

Eqn. (4.58) must be solved, for each iteration, for the change in incremental displacements, $\delta \mathbf{u}_{i+1}$. In order to solve for $\delta \mathbf{u}_{i+1}$ the global stiffness matrix, $\mathbf{K}_G$, must be inverted. Although this is a computationally expensive operation, iteration ensures that a relatively large time increment can be used while maintaining accuracy of solution. Following iteration $i$, $\delta \mathbf{u}_{i+1}$ has been determined and a better approximation of the solution has been made, $\mathbf{u}_{i+1}^{t+\Delta t}$, through Eqn. (4.58). This in turn is used as the current approximation to the solution for the subsequent iteration ($i + 1$).
The accuracy of the method depends on the accurate evaluation of \( G(u_i^{t+\Delta t}) \) for each iteration, which in turn depends on the accurate evaluation of \( \sigma(u_i^{t+\Delta t}) \) (as per Eqn. (4.58)). The latter of these two quantities requires an accurate stress algorithm to be in place to calculate the stresses for each iterative estimate of the displacements, \( u_i^{t+\Delta t} \). The accuracy of the solution is also dictated by the convergence criterion where the updated value for \( G \) must be less than a tolerance value. Complications can arise in an analysis that has a highly non-linear stress-strain response or where there is contact and sliding between two surfaces. For a complex problem it can be difficult to predict how long it will take to solve or even if convergence will occur at all.

4.5 Summary

An overview of the principles of continuum mechanics and finite deformation kinematics are presented in this chapter. The neo-Hookean form of the strain-energy potential for hyperelastic materials is used in this research to represent the PCM and the chondrocyte nucleus. Soft biological tissues such as the ECM in cartilage exhibit anisotropic material behaviour, due to rearrangements in their microstructure, such as reorientation of the collagen fibre directions with deformation. The simulation of these nonlinear effects in this research uses a constitutive model formulated within the framework of anisotropic hyperelasticity. In order to model the mechanical behaviour of the chondrocyte cytoplasm, a theoretical framework for stress fibre remodelling and contractility is used via a user-defined material subroutine (UMAT) (Ronan et al. 2012). The simulations performed in this thesis use the finite element solver Abaqus Standard.
Figure 4.1 Schematic of finite deformation kinematics. The continuum body $V$ is deformed from the reference configuration to the current configuration $V^*$. $\mathbf{x}$ and $\mathbf{y}$ are position vectors defining a particle. The displacement of the particle is given by the vector $\mathbf{u}$. The deformation of an infinitesimal material fibre in the reference and current configurations is denoted by $d\mathbf{x}$ and $d\mathbf{y}$ respectively.
Figure 4.2 (a) Stress fibre formation in response to an exponentially decaying signal \( C \), and generating tension \( T \); (b) cross-bridge cycling tension-velocity relationship for stress fibres. The red solid curve corresponds to a fully activated stress fibre. The blue dashed curve corresponds to a stress fibre that has partially dissociated \( (0 < \eta < 1) \); (c) stress fibre dissociation in response to tension reduction; (d) Cut away view of a 3D cell and nucleus geometry. Insets show schematic of stress fibre distribution on 2D sections at two points in the cell cytoplasm for illustrative purposes (note: distribution is actually predicted in 3D space at every integration point in the cell cytoplasm); (e) the active stress fibre and passive components of the model are placed in parallel and summed to give the total stress. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Chapter 5

5 The Effect of the Cytoskeleton of Single Chondrocytes in the Response to Shear Deformation

5.1 Introduction

Numerous *in vitro* studies have demonstrated that chondrocytes react to mechanical stimuli. Compression of chondrocytes in agarose gel induces disruption of the actin cytoskeleton (Knight et al. 2006; Campbell et al. 2007). In addition, static compression has been shown to downregulate type II collagen expression in chondrocytes (Leipzig and Athanasiou 2008), while cyclic compression restores levels to those of unperturbed cells (Shieh and Athanasiou 2007). Chondrocytes cultured in a monolayer show decreases in chondrogenic gene expression, while inhibition of actin polymerisation causes an increase in type II collagen and glycosaminoglycan production (Woods et al. 2007). Furthermore, disruption of the actin cytoskeleton alters the biomechanical response of chondrocytes to micropipette aspiration (Trickey et al. 2004) and compression (Ofek et al. 2009c). Despite such extensive *in vitro* investigation, the mechanisms by which chondrocytes actively respond to mechanical loading are not well understood.

Previous studies have investigated the effect of shear deformation in cartilage tissue during joint movement (Buckley et al. 2008; Wong and Sah 2010b). It has been demonstrated that elevated shear strains are found in cartilage containing focal defects, suggesting that those strains contribute to the further deterioration of the tissue (Wong and Sah 2010a). Previous *in vitro* studies have shown that shear directly affects chondrocyte morphology (Sawae et al. 2004) and metabolism (Smith et al. 2000). Huang et al. (2003) have shown that as chondrocytes spread with the actin cytoskeleton reorganising at the cell periphery, the cells become more resistant to shear forces. Clearly, shear loading in cartilage is important to investigate. Understanding the role of the actin cytoskeleton in the response of chondrocytes to shear loading may help to elucidate the biomechanisms involved in cartilage regeneration or degeneration.

Finite element modelling has previously been used to characterise the mechanical properties of chondrocytes under mechanical loading, as discussed in
detail in Chapter 2, Section 2.4.1. In summary, elastic, viscoelastic, and biphasic models have been employed to simulate the response of chondrocytes to loading conditions such as compression and micropipette aspiration (Baaijens et al. 2005; Trickey et al. 2006; Ofek et al. 2009b). Of significant importance is a study by McGarry and McHugh (2008), in which a viscoelastic material formulation for the chondrocyte cytoplasm and nucleus is used to simulate the \textit{in vitro} detachment of chondrocytes due to probe indentation (Huang et al. 2003). As previously discussed in Chapter 2, this computational study highlights the shortcoming of passive viscoelastic cell models by demonstrating that the cell stiffness must be artificially increased as cells spread in order to replicate experimental measurements (Huang et al. 2003). The use passive material models do not take into account the key mechanisms by which cells actively respond to mechanical stimuli, and hence offer a limited insight or predictive capability. An active modelling framework proposed by Deshpande et al. (2006), which incorporates the biomechanisms underlying the formation, dissociation and contractility of the actin cytoskeleton has been shown to accurately predict changes in cell contractility as a function of cell area and substrate stiffness (McGarry et al. 2009) for a range of cell phenotypes seeded on micro-pillar arrays. Most recently, this formulation has been shown to accurately predict the increased compressive resistance of spread cells compared to round cells (Ronan et al. 2012). In the current chapter, this formulation is used to demonstrate the important active role of the actin cytoskeleton in the response of chondrocytes to applied shear loading.

The objective of the current chapter is to investigate the role of the active remodelling and contractility of the actin cytoskeleton in the response of chondrocytes to mechanical deformation. Specifically, \textit{in vitro} experiments are performed in which the resistance of single chondrocytes to applied shear deformation is observed. Additionally, tests are also performed on chondrocytes in which the contractile actin cytoskeleton has been disrupted. It is hypothesised that commonly used passive hyperelastic models cannot reproduce the experimentally observed behaviour. An active bio-chemo-mechanical model based on stress fibre evolution and contractility is implemented in order to simulate the \textit{in vitro} shear experiments. It is demonstrated that the active remodelling and contractility of the actin cytoskeleton is the biomechanism governing the response of chondrocytes to

5.2 Experimental Methods

The experimental methods carried out in this chapter have previously been presented in Chapter 3. In the current chapter, probe force-indentation curves were generated for all experimental groups, in order to measure the contribution of the cytoskeleton components in the response of chondrocytes to shear. Probe indentation is defined as the forward-most position of the probe minus the back edge position of the cell.

5.3 Finite Element Implementation

The 3D active modelling framework presented in Chapter 4 is used in the current chapter to represent the chondrocyte cytoplasm. The nucleus is represented as a passive hyperelastic material. Based on previous studies a Young’s modulus of 4 kPa is assumed for the nucleus and a Poisson’s ratio of 0.4 is assumed for both the nucleus and the passive cytoplasm material (Leipzig and Athanasiou 2008; McGarry and McHugh 2008; Ofek et al. 2009a). The substrate and the probe are rigid, as their materials (glass and tungsten, respectively) are several orders of magnitude stiffer than the cell material. Frictionless contact was chosen between the cell surface and the rigid probe. A previously published cohesive zone formulation is used to simulate adhesion between the cell and the rigid substrate (Deshpande et al. 2006). 3D meshes of the cell geometry are based on z-stack images of chondrocytes taken during the in vitro experiments. The cell is modelled using 8-noded linear brick elements (C3D8). 3D meshes of the cell geometry were comprised of approximately
80,000 elements. Mesh sensitivity studies were conducted and it was found that increasing the number of elements beyond 80,000 elements did not increase the accuracy of the simulations.

The simulation consists of two steps. During the initial step, the exponentially decaying signal is initiated allowing the formation of stress fibres in the cell cytoplasm. The step lasts 1000 seconds in order for the stress fibres to reach an equilibrium state. In the second step, the bottom of the probe is moved 4 μm above the substrate using a displacement boundary condition. The probe is then moved horizontally towards the cell at a speed of 4 μm/s using a constant velocity boundary condition. This boundary condition is applied to the top surface of the probe. The computed probe reaction force is plotted against the computed probe indentation for direct comparison with experimental results.

The average stress fibre intensity level, (\(\bar{\eta}\)), is used to show the computed average distribution of actin before and during shear. In the current chapter, the circular variance (\(\Pi\)) is compared to fluorescent images of chondrocytes stained using phalloidin antibodies.

5.4 Results

5.4.1 Experimental Results

A probe force-indentation curve is shown in Figure 5.1 (a) for the untreated cell group. The force increases dramatically upon initial probe indentation. A yield point is reached at 34 nN, after which the force increases gradually with further indentation. A force of 84 nN was measured after 4 μm of probe indentation. Disruption of the actin cytoskeleton using cytochalasin-D (cyto-D) results in a markedly different response compared to untreated cells (Figure 5.1 (a)). Cyto-D treated chondrocytes did not exhibit a rapid increase in force to a yield point. Instead, a linear force-indentation relationship was observed, with forces being significantly lower than untreated cells at all indentation levels (\(p < 0.05\)). At 1 μm of probe indentation, the mean force for cells treated with cyto-D was approximately six times lower than that for untreated cells.
As shown in Figure 5.1 (b), disruption of the non-contractile components of the cytoskeleton (microtubules and intermediate filaments) results in force-indentation curves that exhibit yield points, similar to that for untreated chondrocytes. For both acrylamide cells (intermediate filaments disrupted) and colchicine cells (microtubules disrupted), measured forces are found to be slightly lower than for untreated cells. However, the yield points for acrylamide and colchicine cells are not statistically different to untreated cells. In contrast, there is a statistical difference between the yield point for cyto-D cells compared to acrylamide and colchicine cells ($p < 0.05$). This demonstrates that the distinctive yield point in the probe force-indentation curve occurs due to the contribution of the contractile actin cytoskeleton. A cell height of $11.7 \pm 1.2 \ \mu m$ (mean ± SD) was measured experimentally. The nucleus was found to be $1.72 \pm 0.38 \ \mu m$ (mean ± SD) above the substrate. No statistical difference was observed between untreated cells and cyto-D, acrylamide or colchicine treated cells in terms of cell and nucleus height.

### 5.4.2 Computational Results

In order to investigate the biomechanisms underlying the distinctive yield point in the probe force-indentation curve observed experimentally, the *in vitro* test is simulated using the aforementioned active 3D stress fibre framework. The first step of the analysis entails the simulation of stress fibre evolution during seeding of chondrocytes on a rigid (glass) substrate. Stress fibre formation is driven by an exponentially decaying signal that initiates at the start of the step. Tension is actively generated by these stress fibres, which leads to deformation of the cytoplasm. This cytoplasm deformation leads to a shortening of stress fibres, and an associated tension drop, as described in Eqn. 4.40, which in turn leads to a partial dissociation of the stress fibre.

The predicted evolution of the actin cytoskeleton in response to the exponentially decaying signal is shown at three time-points in Figure 5.2. The mean stress fibre activation level, $\bar{n}$, is shown in Figure 5.2 (a-c). A steady state solution is predicted following 1000 seconds, after which no further changes are computed. At this point, the cell is in equilibrium and all stress fibres in the actin cytoskeleton are at isometric tension with zero fibre strain rate. As can be seen in Figure 5.2 (c), the stress fibre activation level, $\bar{n}$, is highest at the base of the cell, as adhesion of the
cell to the stiff substrate prevents significant tension reduction in this region of the cytoplasm. Similarly, the stiff nucleus also prevents tension reduction in the surrounding cytoplasm, thus leading to a clustering of the actin cytoskeleton around the nucleus.

As can be seen in Figure 5.2 (d-f), the variance parameter $\Pi$ shows that stress fibres are more clustered in a dominant direction at the base of the cell and surrounding the nucleus, following a similar distribution to that of the mean stress fibre activation level $\bar{\eta}$ (Figure 5.2 (a-c)), suggesting that stress fibres are more aligned in a dominant direction at these localised regions in the cytoplasm. However, the maximum value of $\Pi$ in the cytoplasm is computed as 0.4, suggesting that stress fibre alignment is not pronounced or distinct at any point in the cytoplasm.

Figure 5.3 shows the computed cell deformation at three time-points during probe indentation. The distributions of $\bar{\eta}$ (Figure 5.3 (a-c)) and $\Pi$ (Figure 5.3 (d-f)) in the deformed cytoplasm are also shown. Probe indentation leads to a stretching of the cytoplasm at the back of the cell, under the probe (Figure 5.3 (b)). The actin cytoskeleton therefore remains in a state of isometric tension with stress fibres yielding. At the front of the cell the cytoplasm is compressed into the substrate, leading to a shortening of the stress fibres in the actin cytoskeleton, leading to a reduction in stress fibre tension, in accordance with Eqn. 4.40. This tension drop at the front of the cell leads to dissociation of stress fibres in this region, in accordance with Eqn. 4.39. Figure 5.3 (b) and (c) illustrate that stress fibre dissociation occurs at the front of the cell during indentation. A similar trend is predicted for the variance ($\Pi$), with stress fibre bundles dissociating at the front and yielding at the back of the cell (Figure 5.3 (d-f)).

A probe force-indentation curve simulated using the active model is shown in Figure 5.4 (a). An excellent fit with the in vitro experimental data for untreated cells is predicted, with a very similar yield point being computed. This prediction for the active model is achieved using a maximum stress fibre tension of $T_{max} = 0.85\text{kPa}$ and a Hill-type constant of $\bar{K}_v = 6$, with the rate parameters set to $\dot{\epsilon}_0 = 0.003\text{s}^{-1}$, $\theta = 70\text{s}$, $k_f = 10$ and $k_b = 1$. Additionally, the passive Neo-Hookean cytoplasm surrounding the stress fibres is found to have a Young’s modulus of $E_{cyto} = 1.5\text{kPa}$. 
The distinctive yield shape of the experimental and computational probe force-indentation curves can be explained by considering the evolution of stress fibres in the cell. As previously discussed, stress fibres at the front of the cell experience reduced tension during shear deformation, leading to dissociation. Therefore, there is a minimal active contribution from the stress fibres at the front of the cell. At the back of the cell, stress fibres are stretched and hence yield, producing a constant tension equal to the isometric value. Therefore, the distinctive yield shape of the probe force-indentation curve results from the elongation of stress fibres at the back of the cell. It is predicted that further probe penetration leads to a gradual increase in force. This is largely due to the stiff nucleus ($E_{nuc} = 4$ kPa) resisting deformation during loading. As the probe indents the cell, the cytoplasm is deformed and shifted underneath the probe, which results in greater nucleus deformation, and probe forces.

When the active stress fibre component of the model is eliminated, leaving just the passive hyperelastic cytoplasm ($E_{cyto} = 1.5$ kPa), the predicted probe force-indentation curve is linear (Figure 5.4 (b)) and is very similar to the experimentally measured force-indentation curves for cyto-D treated cells. As further highlighted in Figure 5.4 (b), the shape of the force-indentation curve for untreated contractile chondrocytes cannot be simulated by a passive hyperelastic material model. Even if the passive stiffness of the cytoplasm is artificially increased to compute higher probe forces, the resultant “J-type” shape of the force-indentation curve is fundamentally different to the yield-type curve observed experimentally.

A parametric study of the key material constants that govern the contractile behaviour of stress fibres ($T_{max}$ and $k_p$) is presented in Figure 5.4 (c). It is important to note that the distinctive yield type behaviour is predicted for all values of $T_{max}$, indicating that the active model is robust in capturing this behaviour. At the lowest level of contractility considered ($T_{max} = 0.2$ kPa), a reduced yield point of 8 nN is computed, with the probe force-indentation curve slightly higher than the passive hyperelastic probe force-indentation curve ($E_{cyto} = 1.5$ kPa in both models). For higher levels of contractility (e.g. $T_{max} = 2$ kPa), the isometric tension at which stress fibres are yielding is increased. Therefore, more work is done when stress fibres at the back of the cell are elongated as the cell deforms, resulting in an increased probe force. For all values of the Hill-type constant ($\tilde{k}_p$), a distinctive yielding is predicted.
However, the computed yield point is dependent on $k_v$, illustrating the importance of the correct calibration of the Hill-type tension velocity relationship for stress fibre contractility during fibre shortening. This parameter has a significant effect on stress fibre tension reduction and hence fibre dissociation at the front of the cell.

The disruption of non-contractile cytoskeletal components is simulated by lowering the passive Young’s modulus to $E_{cyto} = 0.03$ kPa without changing the active parameters of the model ($T_{max} = 0.85$ kPa). Underlying this strategy is an assumption that the treatment of cells with acrylamide or colchicine will alter the mechanical properties of the passive material surrounding the stress fibres, but will not affect the material properties that govern stress fibre formation and contractility. However, it is important to note that a reduction in the passive stiffness of the cytoplasm will result in a reduced resistance to stress fibre shortening during step one of the analysis. Therefore, a lower steady state distribution of stress fibres will be computed in response to the exponentially decaying signal. This reduction in stress fibre density in the cytoplasm therefore results in an altered probe force-indentation curve during the second step of the analysis. As shown in Figure 5.4 (d), the reduction of the passive cytoplasm stiffness results in the prediction of slightly lower probe forces than computed for untreated cells with a passive cytoplasm stiffness of to $E_{cyto} = 1.5$ kPa (Figure 5.4 (a)). Most importantly, the distinctive yield behaviour of the force-indentation curve is not affected by the reduction in passive stiffness. This is due to the regions surrounding the stiff nucleus and at the base of the cell adjacent to the rigid substrate preventing a reduction in tension despite the lower cytoplasm stiffness. The elongation of stress fibres in these regions results in the distinctive yield shape of the force-indentation curve.

It is also important to note that significantly different stress distributions are computed in the cytoplasm and cell nucleus for an active and passive cell. The stress state predicted by the active cell model in the cell following probe indentation is shown in Figure 5.5 (a). The stress state predicted by the passive hyperelastic cell model is shown for comparison ($E_{cyto} = 1.5$ kPa in both models). Significant differences in cell deformation are computed at the back of the cell. Additionally, considerable differences in the equivalent tensile stress (Von Mises) are predicted, with higher stresses being predicted for the active model. Figure 5.5 (b) highlights
the difference in computed nucleus deformation for the active and passive models. It is computed that the maximum Von Mises stress in the nucleus is 46.7% greater in the case of the active model, with substantially more elongation being predicted due to the tension generated by the elongating stress fibres at the back of the cell.

### 5.4.3 Fluorescent Imaging

Cells, either sheared or control, were subsequently imaged using immunocytochemistry to identify alterations in their intracellular structure, with consistent changes observed among the sheared cells (Figure 5.6). Control cells typically displayed rounded morphologies at all focal planes through the cell’s height, when viewed top-down on the glass slides through the confocal microscope. In contrast, the sheared cells generally sloped downward and became narrower along their trailing side. Focal adhesions were equally distributed throughout the control cells’ periphery, whereas focal adhesions were consistently concentrated along the trailing edge of the cells after shear.

It is observed in the fluorescent microscopy images (Figure 5.7 (a)) that the actin cytoskeleton is uniformly located around the periphery of the base of the cell before shear. A contour plot of the predicted actin cytoskeleton distribution at the base of the cell before shear is plotted in Figure 5.7(b) for comparison with the bottom-up view fluorescence images. The experimental image demonstrates that no distinctive bundling of stress fibres in a dominant direction occurs, instead the actin cytoskeleton has a smeared appearance. This in vitro observation is very close to the predicted distribution using the active model and is mirrored by the low value of $\Pi$ computed. Following probe indentation, it is observed experimentally that the actin cytoskeleton has dissociated at the front of the cell but it remains intact at the back of the cell (Figure 5.7 (c)). The experimentally measured actin intensity at the front of the cell is shown to be significantly lower than in other regions of the cell ($p < 0.5$). Again, this correlates very strongly with the active model as shown in Figure 5.7 (d).

Actin remodelling during shear deformation was quantitatively determined for experimental untreated cells ($n = 5$) by analysing the fluorescent microscopy images. Each cell was divided into four zones as illustrated in Figure 5.8 (a), where zone one is at the front of the cell and zone four is at the back of the cell. The
relative intensity of each zone was determined, and was then divided by the overall relative intensity of the cell to give a normalised relative intensity for each zone. It was found that the area closest to the front of the cell (zone 1) exhibited a decrease in normalised relative intensity (Figure 5.8 (b)). The normalised relative intensity in zone 1 was found to be statistically different from zones 2–4 ($p < 0.009$, $p < 0.0001$, $p < 0.008$), whereas no significant differences were found between zones 2–4.

5.5 Discussion

The current study presents, for the first time, an experimental-computational investigation of the role of the active remodelling and contractility of the actin cytoskeleton in the response of chondrocytes to shear. *In vitro* shear experiments were performed on single chondrocytes, investigating the behaviour both of untreated cells, and of treated cells in which the contractile actin cytoskeleton was removed. A 3D active modelling framework, incorporating signal dependent stress fibre formation and tension-dependent stress fibre dissociation, was used to simulate the *in vitro* experiments. Simulations elucidate the important role of the actin cytoskeleton in chondrocytes during shear deformation. In particular, this chapter uncovers the following: (i) *in vitro* experiments reveal a distinctive yield shape for the force probe-indentation curve; (ii) in contrast, a linear probe force-indentation curve is observed experimentally for cells in which the active contractile cytoskeleton is removed; (iii) the active modelling framework provides a highly accurate prediction of the response of untreated chondrocytes to shear deformation, capturing the distinctive yield point in the probe force-indentation curve; (iv) a passive hyperelastic model is incapable of predicting the response of untreated chondrocytes during shear, and is only found to provide an accurate prediction for cells in which the actin cytoskeleton has been disrupted; (v) experimentally, disruption of the intermediate filaments and microtubules did not have a significant effect on the response of the cell to shear, with the distinctive yield shape still being observed; (vi) by reducing the passive stiffness of the material that surrounds stress fibres, the active modelling framework provides an accurate prediction of the behaviour of chondrocytes in which the intermediate filaments and microtubules have been disrupted.
The current study, for the first time, uses a 3D implementation of the active modelling framework to investigate the response of chondrocytes to shear loading. Shear loading is particularly interesting as it imposes both tensile and compressive strain to separate regions within the cell which leads to very different regimes of stress fibre behaviour. The current study demonstrates the importance of firstly predicting where stress fibres form in the cell, and then the subsequent response of stress fibres to shear deformation. Stress fibre formation is initiated by an exponentially decaying signal within the cell. A steady state equilibrium distribution of the actin cytoskeleton is predicted to occur, with stress fibres predicted predominantly at the base of the cell and surrounding the stiff nucleus. During subsequent shear deformation, the cytoplasm at the front of the cell is compressed into the substrate, leading to a shortening of stress fibres in this region, and as a consequence, tension reduction and localised dissociation is predicted. At the back of the cell, there is no reduction in tension and therefore stress fibres stretch at isometric tension and remain intact. The yield point in the force-indentation curve is a direct result of the distinctive behaviour of the actin cytoskeleton in compressive and tensile regions of the cell, and can only be predicted by an active modelling framework that incorporates the remodelling and contractility of the actin cytoskeleton. Previous computational models of chondrocytes have relied on passive material formulations (Baaijens et al. 2005; Ofek et al. 2009a; Nguyen et al. 2010), ignoring the key biomechanical features underlying stress fibre formation, remodelling, and contractility. A number of previous in vitro studies have considered the role of the actin cytoskeleton in chondrocytes in response to mechanical loading. Knight et al. (2006) have demonstrated that actin filaments in chondrocytes dynamically adapt to compression and hydrostatic pressure. Additionally, it has been reported that actin filaments contribute significantly to cell stiffness during static compression (Leipzig et al. 2006). It has also been shown that the disruption of the actin cytoskeleton results in a significant reduction in the stiffness of chondrocytes during compression compared to disruption of intermediate filaments and microtubules (Ofek et al. 2009c). The current study, for the first time, provides a quantification and interpretation of the importance of the active contractility of the actin cytoskeleton in chondrocytes during shear.
While the current chapter highlights the importance of the actin cytoskeleton
in the biomechanical behaviour of chondrocytes as discussed above, it is important
to note that the actin cytoskeleton is quite smeared in appearance, without distinctive
bundling of stress fibres. This smeared appearance of the chondrocyte actin
cytoskeleton is in agreement with previously published in vitro images of non-
passaged chondrocytes (Idowu et al. 2000; Trickey et al. 2004; Leipzig et al. 2006),
and chondrocytes embedded in an extracellular matrix in situ (Durrant et al. 1999;
Langelier et al. 2000). The active modelling formulation used in the current chapter
has been used to simulate the contractility of smooth muscle cells, mesenchymal
stem cells, and fibroblasts seeded on micro-posts (McGarry et al. 2009), and during
compression (Ronan et al. 2012). McGarry et al. (2009) determined the maximum
isometric tension ($T_{\text{max}}$) for smooth muscle cells ($T_{\text{max}} = 25$ kPa), mesenchymal
stem cells ($T_{\text{max}} = 8$ kPa), and fibroblasts ($T_{\text{max}} = 3.25$ kPa). However, these values
are much higher than the value calibrated for chondrocytes in the current chapter
($T_{\text{max}} = 0.85$ kPa). In the current chapter simulations predict a low value for stress
fibre variance in the chondrocyte cytoplasm ($\Pi_{\text{max}} \approx 0.4$), indicating that highly
aligned stress fibre bundles are not formed for this cell phenotype, which is in
agreement with the experimentally observed smeared actin cytoskeleton. In contrast,
the stress fibre variance in smooth muscles cells ($\Pi_{\text{max}} \approx 0.8$), mesenchymal stem
cells ($\Pi_{\text{max}} \approx 0.8$), and fibroblasts ($\Pi_{\text{max}} \approx 0.8$) is very high, predicting stress fibres
align in dominant directions for these cell phenotypes (Ronan et al. 2012). However,
despite the absence of highly aligned distinctive stress fibres in chondrocytes, the
current chapter unambiguously demonstrates the significant contribution of the
smeared actin cytoskeleton to the biomechanical behaviour of chondrocytes.

The active modelling framework used in the current chapter provides a
prediction of actin cytoskeleton distribution and contractility in chondrocytes, which
in turn causes stress in the cell nucleus. Hence, even in the absence of an externally
applied load, stresses will be actively generated by the remodelling actin
cytoskeleton, as predicted in the first step (prior to probe indentation) of the analyses
presented in the current chapter. In contrast, passive models will not provide a
prediction of cell stress generated by a contractile remodelling actin cytoskeleton,
hence cytoplasm and nucleus stresses will only be computed in response to an
externally applied load. For example, in the current chapter the stresses computed in
the nucleus when using a passive model are entirely due to the indentation of the probe. It is important to note that following probe indentation, the cytoplasm and nucleus stress state computed using the active stress fibre formulation is considerably different to that computed by the passive cell model. The accurate prediction of nucleus stresses is particularly important in light of in vitro studies that have demonstrated a link between nucleus deformation and gene expression (Thomas et al. 2002; Roca-Cusachs et al. 2008). An experimental study (1997) has demonstrated that connections between the cytoskeleton and the nucleus provide a path for signal transfer in cells, and that cellular deformations may lead to changes in DNA structure. Nucleus deformation in chondrocytes has been examined experimentally (Guilak 1995; Guilak et al. 2000), and simulated using passive material models (Vaziri and Mofrad 2007; Finan et al. 2009). The active modelling framework in the current chapter has the potential to elucidate the link between active contractility, nucleus deformation and mechanotransduction in chondrocytes.

Actin cytoskeleton dissociation in compressive regions of the cytoplasm is particularly important for chondrocyte mechanotransduction. For instance, the disruption of the actin cytoskeleton in chondrocytes embedded in an agarose gel during cyclic compression has been reported (Knight et al. 2006; Campbell et al. 2007). Such compression induced dissociation of the actin cytoskeleton has important implications for the tissue engineering of cartilage. Shieh and Athanasiou (2007) have reported that the dynamic compression of single chondrocytes leads to an up-regulation of aggrecan and type II collagen, but static compression causes a down-regulation. The findings of the current chapter can provide an interpretation of previously reported trends for in vitro chondrocyte compression experiments. The active modelling framework suggests that cyclic tension reduction, as occurs in dynamic compression experiments, will lead to greater dissociation of the actin cytoskeleton than a single static compression load. The importance of the actin cytoskeleton in the tissue engineering of cartilage is further highlighted by Woods et al. (2007) in which chemical disruption of the actin cytoskeleton results in an up-regulation of type II collagen and glycosaminoglycans. An application of dynamic boundary conditions to the 3D active modelling framework presented in the current chapter could potentially identify optimal mechanical loading conditions for the design of tissue-engineered cartilage constructs.
The focus of the current chapter on shear deformation of single chondrocytes is of particular physiological relevance, given the established links between shear loading and pathological cartilage tissue. Wong and Sah (2010a) have shown that shear strain is dramatically altered in patellar cartilage due to focal defects. In addition, shear strain is significantly increased in degenerated cartilage and this outcome is amplified with reduced lubrication (Wong et al. 2008). Smith et al. (2004) have demonstrated that shear stress decreases aggrecan and collagen type II expression in osteoarthritic chondrocytes. Additionally, shear stress causes an increase in nitric oxide production in osteoarthritic chondrocytes leading to increased cell apoptosis (Lee et al. 2002). The effect of altered shear strains due to focal defects or deteriorated tissue on cytoskeleton remodelling and contractility in chondrocytes in situ is investigated in the next chapter.

In the current chapter, results are presented only for probe indentations that did not lead to bond rupture between the cell and the substrate. The absence of bond rupture was confirmed by fluorescent imaging of the focal adhesion protein vinculin, which was observed at the periphery of the cell. Previous in vitro experiments have investigated cell adhesion and detachment during shear deformation (Hoben et al. 2002; Huang et al. 2003). A computational study (McGarry and McHugh 2008), based on the experimental findings of Huang et al (2003), illustrates that a strengthening of the cell-substrate interface in tandem with cytoplasm stiffening during cell spreading results in increased cell detachment forces. Experimentally, it has been demonstrated that focal adhesion formation is sensitive to external mechanical stimulation (Sniadecki et al. 2007). Also, the relationship between focal adhesion area and active cell contractility has been reported (2003). Following from the experiments presented in the current chapter and those of Huang et al. (2003), a series of in vitro tests should be performed in which focal adhesion evolution is examined as a function of probe indentation and actin cytoskeletal contractility. This would provide insight into the important link between the formation of focal adhesions and tractions at the cell-extracellular matrix interface due to internal contractility and external applied loading.

In conclusion, this chapter illustrates the importance of the actin cytoskeleton in the shear deformation of chondrocytes. In vitro shearing of single cells reveals a
characteristic yield point in the force-indentation curve for untreated chondrocytes. Simulations using an active 3D framework for stress fibre remodelling and contractility reveal for the first time the importance of the actin cytoskeleton in the biomechanics of chondrocytes. The distinctive force-indentation curve results from the yielding of the stress fibres in localised tensile regions at the back of the cell, in tandem with the dissociation of stress fibres in localised compressive regions at the front of the cell. Importantly, in vitro disruption of the actin cytoskeleton results in a linear force-indentation relationship, in contrast to the yield-type relationship observed for untreated chondrocytes. It is demonstrated that passive hyperelastic cell models can only be used to predict the response of non-contractile cells to shear deformation. The present chapter demonstrates that the simulation of the active cellular biomechanisms is critical in order to provide accurate predictions of the response of cells to mechanical stimuli. Furthermore, the current chapter presents, for the first time, a novel computational-experimental methodology for the calibration of the active model in which active and passive cell behaviour has been parsed. The combined modelling-experimental framework uncovers the contribution of active contractility and remodelling of the actin cytoskeleton to the response of chondrocytes to shear loading, with important implications for the understanding of the pathogenesis of cartilage tissue and the tissue engineering of cartilage constructs.
References


Figure 5.1 Experimental probe force-indentation data for: (a) untreated and cyto-D cells; (b) untreated, acrylamide and colchicine cells. The data points represent the average force and indentation values for the experimental groups (mean ± SD). Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Figure 5.2 Contour plots of the average stress fibre activation levels ($\bar{\eta}$): (a) at 30 s after signal initiation; (b) at 70 s after signal initiation; (c) at 1000 s after signal initiation. Contour plots of the variance ($\Pi$): (d) at 30 s after signal initiation; (e) at 70 s after signal initiation; (f) at 1000 s after signal initiation. A half-cell is shown due to symmetry. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.

Figure 5.3 Contour plots of the average stress fibre activation levels ($\bar{\eta}$) at a probe indentation of: (a) 1.5 µm; (b) 4.7 µm; (c) 10.9 µm. Contour plots of the variance ($\Pi$) at a probe indentation of: (d) 1.5 µm; (e) 4.7 µm; (f) 10.9 µm. A half-cell is shown due to symmetry. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Figure 5.4 (a) Computational probe force-indentation curves for the active model \( \left( E_{cyto} = 1.5 \text{ kPa}, T_{max} = 0.85 \text{ kPa}, \bar{k}_p = 6 \right) \), mean experimental untreated cell data included for comparison. (b) Computational force probe-indentation curves assuming a passive hyperelastic cell cytoplasm. Predictions are shown for three values of cytoplasm stiffness: 1.5 kPa, 4 kPa, and 8 kPa. (c) Parametric study of the effect of active parameters \( T_{max} \) and \( \bar{k}_p \) on predicted probe force-indentation curves (with \( E_{cyto} = 1.5 \text{ kPa} \)). (d) Computational probe force-indentation curve for the active model with reduced cytoplasm stiffness \( \left( E_{cyto} = 0.03 \text{ kPa}, T_{max} = 0.85 \text{ kPa}, \bar{k}_p = 6 \right) \), and experimental probe force-indentation data (mean ± SD) for acrylamide and colchicine treated cells. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Figure 5.5 (a) Contour plots of the equivalent tensile (Von Mises) stress in the cell predicted by the active (left) and passive (right) models; (b) contour plots of Von Mises stress in the nucleus predicted by the active (left) and passive (right) models. All stresses are computed after 12 µm of probe indentation. The probe and substrate are removed from the image for clarity. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Figure 5.6 Alterations in cell structure in response to direct shear. The bright field image, nuclei (displayed in blue), actin network (displayed in red), and focal adhesions (displayed in green) are shown for a representative control and sheared cell at different focal planes in the cell’s height. A clear change in cellular morphology and intracellular organisation of actin and focal adhesions can be observed for the cell experiencing the shear force. The control cell exhibits a
rounded morphology when viewed top down on the glass slides with actin and focal adhesions equally distributed along its periphery at all focal planes. In contrast, the sheared cell undertakes a ‘sloped’ morphology along its trailing side, which converges akin to a ‘comet’s tail’. The actin network in the sheared cell can be identified concentrated close to the cell’s base, on the trailing side of the cell directly behind the nucleus. In addition, strong pockets of focal adhesions can be observed beneath the back edge of the cell, near its base. Reproduced with permission from Ofek et al. 2009, BMMB, 9(2): 153-162.

Figure 5.7 (a) Representative brightfield and fluorescent image (bottom-up view) of a cell before shear deformation, with nuclei (blue) and actin (red) shown at a focal plane near the base; (b) Predicted distribution of the actin cytoskeleton before shear deformation for the active model ($E_{cyto} = 1.5$ kPa, $T_{max} = 0.85$ kPa, $k_v = 6$); (c) Representative brightfield and fluorescent image (bottom-up view) of a cell after shear deformation; (d) Predicted distribution of the actin cytoskeleton following 10.9 µm of probe indentation. The arrow indicates the direction of probe movement. Reproduced with permission from Dowling et al. 2012, J R Soc Interface, 9(77):3469-79.
Figure 5.8 (a) Bright field image of a representative cell (bottom-up view) with sub-cellular zones depicted. The cell is split into four zones, with zone 1 located at the leading edge of the cell and zone 4 located at the trailing edge of the cell. The arrow indicates the direction of probe movement; (b) normalised relative intensity of actin for cells after shear deformation ($n = 5$) determined by fluorescence microscopy. Results are presented as normalised relative intensity ± SD for each sub-cellular zone. * indicates statistical significance of zones 2, 3 and 4 over zone 1 ($p < 0.05$).

6 In Vitro Chondrocyte Detachment

6.1 Introduction

Chondrocyte adhesion is a key phenomenon that affects fundamental cellular processes such as morphology, migration, cell-signalling and differentiation (Dürr et al. 1993; Frenkel et al. 1996; Darling and Athanasiou 2005; Kurtis et al. 2006; Chen et al. 2012). Additionally, the ability of cells to attach to scaffold materials can significantly affect the performance of tissue engineered cartilage (Ishaug-Riley et al. 1999; Li et al. 2006). Furthermore, chondrocyte adhesion is important for wound healing in articular cartilage including the repair of focal defects (Buckwalter and Mankin 1998; Lyman et al. 2012).

Numerous in vitro studies have investigated the biomechanical response of chondrocytes during adhesion and detachment. A study by Huang et al. (2003) measured the force required to detach in vitro single chondrocytes adhered to glass substrates using a horizontally moving probe over a time period of six hours. It was shown that the detachment force increases significantly from 34 nN for a one hour round cell to 388 nN for a six hour cell (Table 6.1). Interestingly, it was observed that the actin cytoskeleton reorganised to the cell periphery during cell spreading. A similar increase in resistance to cell detachment during cell spreading was also observed when chondrocytes were exposed to fluid-induced shear stress (Schinagl et al. 1999). Genes et al. (2004) have shown that chondrocytes attach more rapidly and to a greater extent on stiff substrates and display a flattened morphology containing stress fibres, whereas chondrocytes on less-stiff surfaces are typically spherical with punctuate actin. Additionally, substrate material has been shown to affect shear detachment forces for fibroblasts when indented using a laterally moving cantilever (Yamamoto et al. 2000). Despite such extensive in vitro investigation, the biomechanisms involved during the detachment of cells, and in particular chondrocytes, is poorly understood.

Finite element modelling has previously been used to simulate the behaviour of cells during detachment (Shin and Athanasiou 1999; McGarry et al. 2005; Cheng et al. 2009). Of particular interest is the study of McGarry and McHugh (2008),
where a viscoelastic material model for the chondrocyte cytoplasm was employed to simulate the in vitro study of Huang et al. (2003). It was shown that approximately an 18-fold increase in the stiffness of the cell and a 4-fold increase in the cell-substrate interface strength was required in order to replicate the experimental measurements of Huang et al. (2003). As previously discussed in Chapter 2, the simulations of McGarry and McHugh (2008) demonstrate that passive models can only capture experimental behaviour by artificially modifying cell material properties and cell-substrate interface properties, providing limited insight into the underlying mechanisms involved in fundamental processes such as cell spreading and detachment.

The objective of the current chapter is to determine whether the active contractility and remodelling of the actin cytoskeleton can account for the increased resistance of spread chondrocytes to detachment, as observed in the in vitro study of Huang et al. (2003). Specifically, an active formulation based on the remodelling and contractility of the actin cytoskeleton is implemented to simulate the biomechanical behaviour of chondrocytes in tandem with a cohesive zone model to simulate the cell-substrate interface. It is demonstrated that the highly developed contractile actin cytoskeleton plays a key role in the increased detachment resistance of spread cells.

6.2 Methods

6.2.1 Cohesive Zone Formulation

A passive cohesive zone model is implemented at the cell-substrate interface to simulate shear detachment where the shear traction-separation relationship is given as:

\[
\tau = \tau_{\text{max}} \exp(1) \left( \frac{\Delta_t}{\delta_t} \right) \exp \left( - \sqrt{\frac{\Delta_t^2}{\delta_t^2}} \right)
\]  

(6.1)

where \(\tau\) is the shear traction, \(\tau_{\text{max}}\) is the critical interface strength, \(\Delta_t\) is the shear separation and \(\delta_t\) is the characteristic interface length. Normal separation is assumed not to occur in response to applied shear deformation of the cell, as demonstrated in
the study of McGarry and McHugh (2008). In summary, interface tractions ($\tau$) increase during relative displacement of the adhered surfaces (cell-substrate) until a critical strength is reached ($\tau_{max}$), after which debonding initiates. Further separation leads to a reduction in traction until full separation occurs. The shear traction-separation curve for the cell-substrate interface is shown in Figure 6.1 (a). A characteristic length ($\delta_c$) of 30 nm is assumed for all simulations based on reported lengths of ligand-receptor bonds (Leckband and Israelachvili 2001). A similar cohesive zone formulation has previously been used to simulate the cell-substrate interface during the detachment of passive viscoelastic chondrocytes (McGarry and McHugh 2008).

6.2.2 Finite Element Implementation

3D finite element meshes of three hour (3 hr) and six hour (6 hr) cell geometries are shown in Figure 6.1 (b) and (c). The 3 hr cell is based on z-stack images of in vitro chondrocytes taken during the experimental study presented in Chapter 3. The finite element model of the 6 hr cell is reconstructed from measurements of a 6 hr cell from the study of Huang et al. (2003), in which the cell contact area and height was measured. The nucleus geometry and position for the 6 hr cell were based on observations from previous studies (Caille et al. 2002; McGarry and Prendergast 2004; Jean et al. 2005).

The following parameters for the active model are used in the current chapter, based on the previous calibration of single chondrocytes presented in Chapter 5, where: $T_{max} = 0.85$ kPa, $\bar{k}_p = 6$, $\bar{\varepsilon}_0 = 0.003$ s$^{-1}$, $\theta = 70s$, $k_f = 1$; $k_f = 10$; $E_{cyto} = 1.5$ kPa. $\nu_{cyto} = 0.4$. Again, similar to Chapter 5, the nucleus is represented as a passive hyperelastic material with $E_{nuc} = 4$ kPa and $\nu_{nuc} = 0.4$. The substrate (glass) and the probe (tungsten) are modelled as rigid bodies. The cohesive zone model is implemented in the finite element code (Abaqus 6.11; Simulia, RI, USA) via a user-defined interface (UINTER). The cell is modelled using 8-noded linear brick elements (C3D8). 3D meshes of the 3 hr and 6 hr cell geometries were comprised of approximately 80,000 and 95,000 elements respectively. Mesh sensitivity studies were performed and no changes to the results were observed when mesh densities were increased.
The simulation consists of two steps. The first step allows the formation of the actin cytoskeleton in the cell cytoplasm in response to a signal. In the second step, the probe is moved 1 µm above the substrate and it is then moved laterally towards the cell at a speed of 1 µm s\(^{-1}\). Displacement and velocity boundary conditions are used to control the height and speed of the probe respectively.

6.3 Results

6.3.1 Probe Force-Indentation Behaviour

As implemented in Chapter 5, an equilibrium actin cytoskeleton distribution is determined by the application of an exponentially decaying signal. The same material properties are used for both the 3 hr and 6 hr cell, with the only difference being the cell geometries, which are based on the observations of Dowling et al. (2012) and Huang et al. (2003). The predicted distribution of the actin cytoskeleton for the 3 hr and 6 hr cell geometries at a steady state solution is shown in Figure 6.2 (a) and (b) respectively. It should be noted that the nucleus is not shown in Figure 6.2 ((a) – (d)) in order to provide an enhanced view of the actin cytoskeleton distribution. In both cases the highest values for the mean stress fibre activation level (\(\bar{\eta}\)) are at the base of the cell and surrounding the nucleus. This is due to the presence of a stiff nucleus and the attachment of the cell to a rigid substrate, both of which supports tension in the stress fibres. The 6 hr cell displays significantly higher levels of stress fibre formation throughout the cytoplasm, particularly above and below the nucleus compared to the 3 hr cell. The area of adhesion of the 6 hr cell to the rigid substrate is greater than that of the 3 hr cell by a factor of two. Additionally the flatter geometry of the 6 hr cell is less deformable than the rounded geometry of the three hour cell. The larger area of adhesion and the stiffer structure of the 6 hr cell provides greater support for stress fibre tension, hence a more highly developed actin cytoskeleton is computed throughout the cytoplasm of the 6 hr cell in comparison to the 3 hr cell, as characterised by the distribution of the mean stress fibre activation level \(\bar{\eta}\). In addition to greater levels of actin cytoskeleton formation, the actin cytoskeleton is also found to be more aligned in dominant directions in the 6 hr cell, as characterised by the higher level of the variance parameter \(\Pi\) (where \(\Pi = \eta_{\text{max}} - \bar{\eta}, 0 \leq \Pi \leq 1\)) computed throughout the cytoplasm of the 6 hr cell (Figure 6.2 (c)) in
comparison to the 3 hr cell (Figure 6.2 (d)). However, as can be noted from the maximum value of $\Pi = 0.45$, the cytoskeleton is not predicted to form highly unidirectional stress fibres, even for the 6 hr geometry.

Figure 6.3 shows the predicted cell deformation after 6 $\mu$m of probe indentation for the 3 hr and 6 hr, with the distributions of $\bar{\eta}$ and $\Pi$ also shown. Again, the nucleus is not shown in Figure 6.3 ((a) – (d)) in order to provide an enhanced view of the actin cytoskeleton distribution. For all simulations, the probe is positioned at a height of 1 $\mu$m above the substrate. It is predicted that the cytoplasm is stretched at the back of the cell during probe indentation, while at the front of the cell the cytoplasm is compressed into the substrate (similar to the trends reported in Chapter 5). Again, tensile strain imposed on the back of the cell results in the actin cytoskeleton remaining intact at a tension equal to isometric value, providing resistance to deformation. In contrast, the compressive strain imparted on the front of the cell leads to a reduction in tension and dissociation of the actin cytoskeleton, in accordance with Eqs. 4.39 and 4.40. As shown in Figure 6.3 (a) and (b), the mean activation level of the actin cytoskeleton ($\bar{\eta}$) reduces throughout the cytoplasm during indentation, with the exception of a small region at the back of the cell. Interestingly, the degree of alignment of the actin cytoskeleton does not significantly alter during probe indentation of the 6 hr cell (Figure 6.3 (d)). While the exact probe height used in the experiments of Huang et al (2003) is not reported, Figure 6.4 demonstrates that a probe height of 4 $\mu$m, as implemented in Chapter 5 for a 3 hr cell, fails to detach the 6 hr cell. Hence it is assumed that the detachment tests of Huang et al. (2003) were performed at a lower probe height.

In order to investigate the effect of increased resistance to applied shear deformation for spread cells, an initial simulation is performed in order to compare the probe forces for an active and passive model where detachment is not simulated. Probe force-indentation curves for the 3 hr and 6 hr cells simulated using a passive hyperelastic model and the active model are shown in Figure 6.5. For the passive model, there is only a slight difference in the probe force-indentations curves for the 3 hr and 6 hr cell. At a probe indentation of 4 $\mu$m, the probe force computed for the 6 hr cell is 11 nN higher than the probe force for the 3 hr cell. In contrast, larger differences are computed for the 3 hr and 6 hr cells when the active model is used.
At a probe indentation of 4 µm, the probe force for the 6 hr cell is 35 nN higher than for the 3 hr cell. Once again, the characteristic yield point in the force-indentation curve can be noted for both the 3 hr and 6 hr geometries at a probe height of 1 µm, which is similar to that reported for the 3 hr cell at a probe height of 4 µm, as shown in Chapter 5.

In order to simulate detachment of the cell from the substrate during applied shear deformation, a cohesive zone formulation (Eqn. 6.1) is implemented at the cell-substrate interface so that bond rupture will occur when a critical interface stress ($\tau_{\text{max}}$) is reached. The effect of varying the critical interface strength for both cell geometries is investigated in order to determine the value of interface strength that provides the closest match to the experimentally measured values of Huang et al. (2003). Figure 6.6 shows probe force-indentation curves for the 3 hr and 6 hr cells with an active contractile actin cytoskeleton, and interface strengths ranging from 0.05 to 0.2 kPa. As shown in Figure 6.6 (a), the initial stages of the force-indentation curves (including the yield point) for the 3 hr cell is not dependent on the interface strength. However, an increase in interface strength results in an increase in the level of probe indentation required to cause cell detachment, and consequently a higher detachment force. For an interface strength of 0.05 kPa, detachment occurs at 5.4 µm of probe indentation at a force of 91 nN, whereas in the case of the highest interface strength of 0.2 kPa, detachment occurs at a probe indentation of 8.5 µm and a force of 150 nN. This range of detachment forces correlate well with the experimentally measured values (Huang et al. 2003) of 72 ± 14 nN and 183 ± 21 nN for a 2 hr and 4 hr cell respectively.

As shown in Figure 6.6 (b), the initial yield behaviour of the 6 hr cell is considerably affected by the interface strength. For interface strength values lower than 0.1 kPa, the maximum detachment forces computed are lower than the forces predicted for the 3 hr cell. However, increasing the interface strength to 0.2 kPa results in a detachment force of 304 nN at probe indentation of 11.25 µm, which is approaching the experimentally measured value of 388 ± 78 nN (Huang et al. 2003). Additionally, it is interesting to note that when the active component of the model is removed, leaving just the passive hyperelastic cytoplasm, the computed detachment force for the 3 hr cell is 40 nN for an interface strength of 0.2 kPa (Figure 6.6 (a)).
This detachment force is similar to that measured for a 1 hr cell (34 ± 18 nN) in the Huang et al. (2003) study, suggesting that the actin cytoskeleton hasn’t significantly developed in a cell spread for 1 hr. Peak detachment forces are reproduced in Figure 6.6 (c) for clarity.

### 6.3.2 Evolution of Interface Traction

As previously mentioned, the initial yield behaviour predicted in the probe force-indentation curves is affected by the interface strength for the 6 hr cell, but not for the 3 hr cell. This difference in behaviour between the two cell geometries can be explained by considering the evolution of interface tractions at the base of the cell during probe indentation. Figure 6.7 shows contours of the traction at the base of the cell during the debonding process for the 3 hr cell with an interface strength of 0.1 kPa. Initial bond rupture occurs at a thin strip at the very front edge of the cell base when the critical interface stress is reached after 6.89 µm of probe penetration (Figure 6.7 (a)). Interestingly, this region of the cell is not located under the probe. Peak tractions are then predicted to occur in the middle and the back of the cell (Figure 6.7 (b)). Further probe penetration causes all remaining adhered nodes under the probe to debond, causing the back of the cell to slide (Figure 6.7 (c)). Peak tractions are reached at the front of the cell at 6.97 µm of probe penetration (Figure 6.7 (d)), which finally results in the detachment of the cell. The most important observation from Figure 6.7 is that the debonding process for the 3 hr cell is very rapid, with initial bond rupture occurring at 6.89 µm of probe indentation, followed by complete detachment at 6.97 µm. This pattern of detachment for the 3 hr cell is predicted for all interface strengths simulated in this chapter, with the only disparity being the level of indentation at which this pattern initiates.

At low values of interface strength (0.05 and 0.075 kPa) for the 6 hr cell, tractions reach the critical stress at the back of the 6 hr cell after an initial probe indentation of 0.26 µm as shown in Figure 6.8. Following debonding of the nodes at the back of the cell, the location of peak interface tractions move from the back of the cell base towards the front (Figure 6.8 (b) and (c)). With the back of the cell sliding along the substrate, further probe indentation leads to peak tractions being reached in the remaining adhered nodes at the front of cell (Figure 6.8 (d)), resulting in complete detachment at a probe indentation of 5.17 µm. It is important to note that
at low values of interface strength for the 6 hr cell, the debonding process occurs at probe indentations lower than that predicted for the 3 hr cell. However, detachment is incremental for the 6 hr cell, compared to the rapid process predicted for the 3 hr cell.

Increasing the interface strength to 0.02 kPa for the 6 hr cell significantly alters the pattern of cell detachment and the level of indentation needed for debonding (Figure 6.9). A region of increased traction develops in the middle of the cell under the probe at a very high level penetration of 11.15 µm, leading to bond rupture (Figure 6.9 (a)). Further probe indentation leads to peak tractions spreading radially throughout the base of the cell (Figure 6.9 (b)) until only the front and back edges of the cell are adhered to the substrate (Figure 6.9 (c)). In contrast to Figure 6.7 and 6.7, where the back of the cell slides along the substrate while the front is still attached, the last region of nodes in the 6 hr cell (with the highest interface strength) to debond is located at the back edge of the cell under the probe (Figure 6.9 (d)). It is important to note that a high level of probe indentation (12.97 µm) is required to detach the 6 hr cell when an interface strength of 0.2 kPa is chosen. In addition, a ‘critical’ interface strength is predicted for the 6 hr cell during probe indentation, in which an interface strength above this threshold value will not affect the yield behaviour of the cell. In contrast, an interface strength value below the threshold limit dramatically alters the yield point.

6.4 Discussion

The current chapter presents a computational investigation of the role of the remodelling and contractility of the actin cytoskeleton in the response of round and spread chondrocytes during *in vitro* shear detachment. Finite element models of cells seeded on rigid (glass) substrates for 3 and 6 hrs are created based on experimentally measured cell geometries (Huang et al. 2003; Dowling et al. 2012). A 3D active modelling framework, based on signal-dependent stress fibre formation and tension-dependent stress fibre dissociation is implemented to predict the distribution of the actin cytoskeleton in the chondrocyte cytoplasm. Additionally, a cohesive zone model is used to simulate debonding at the cell-substrate interface. The present chapter demonstrates that the active formulation combined with a cohesive zone model, using an interface strength of 0.2 kPa, provides a reasonable agreement with
the measurements of Huang et al. (2003). The experimental study of Huang et al. (2003) shows that the force required to detach a cell spread for 6 hrs was $388 \pm 78$ nN, while 304 nN is predicted in the current chapter for an interface strength of 0.2 kPa. Additionally, for the same interface strength, the detachment force for a 3 hr cell is computed to be 150 nN, compared to 72 ±14 nN and 183 ± 21 nN for a 2 hr and 4 hr cell respectively. This illustrates the important role of the actin cytoskeleton in detachment resistance of cells. This is further outlined in a simulation which removes the active contribution of the cytoplasm, and predicts an extremely low detachment force of 40 nN. As previously mentioned, the study of Huang et al. (2003) shows that the detachment force is $34 \pm 18$ nN for a 1 hr spread cell. The chapter suggests that this very low detachment force for a 1 hr cell is because a significant assembly of the actin cytoskeleton has not occurred.

The simulations presented in the current chapter demonstrate that an active modelling framework which incorporates the contractile actin cytoskeleton can capture the response of chondrocytes during *in vitro* shear detachment. Numerous finite element simulations have used passive material models in order to capture the behaviour of cells in response to mechanical stimuli. Caille et al. (2002) have shown that an artificial increase in cell stiffness is needed in order to capture the increased resistance to deformation during applied compression for single endothelial cells. A similar artificial increase in material properties is required for simulations of round and spread fibroblasts subjected to micropipette aspiration (Thoumine et al. 1999). The computational study of McGarry and McHugh, also based on the experimental findings of Huang et al., shows that an increase in cell stiffness in tandem with an increase in the cell-substrate interface strength must be artificially implemented for spread chondrocytes in order to compute detachment forces similar to experimental values. In contrast, the current chapter shows that using an interface strength of 0.2 kPa provides a reasonable prediction of the detachment forces for both the 3 hr and 6 hr cells. Additionally, the active modelling formulation implemented in this chapter uses an unchanged set of material parameters. These parameters have been shown to provide a strong correlation between experimental results and simulations for *in vitro* single chondrocytes during shear deformation (Chapter 5). The active modelling formulation demonstrates the ability to capture the trend of increased detachment forces as observed experimentally by Huang et al. (2003), indicating that the
increased resistance to shear detachment is mainly due to the highly developed actin cytoskeleton in spread chondrocytes. Furthermore, there is no requirement to adjust the material properties of the cell or the interface strength in the present chapter.

The current chapter demonstrates that a more highly developed actin cytoskeleton is predicted for the 6 hr cell geometry compared to the 3 hr cell geometry. This is due to the 6 hr cell having a larger area of adhesion and being less deformable than the 3 hr cell, thus providing greater support for stress fibre tension and preventing dissociation of the actin cytoskeleton. Additionally, the actin cytoskeleton is found to be more aligned in dominant directions in the 6 hr cell. In the study of Huang et al. (2003), an increase in cell contact area and a decrease in cell height was observed during the 6 hrs of seeding. Interestingly, fluorescence microscopy showed that the actin cytoskeleton rearranged from the centre of the cell to the periphery during spreading. It has been shown experimentally that when chondrocyte morphology is rounded, actin filaments are distributed in a punctuate manner containing no distinctive bundling of stress fibres (Figure 2.6 and 2.9) (Durrant et al. 1999; Idowu et al. 2000; Langelier et al. 2000; Knight et al. 2001; Trickey et al. 2004; Leipzig et al. 2006; Ofek et al. 2009). In contrast, chondrocytes that have spread for several hours develop a flattened fibroblast-like morphology with highly aligned stress fibres throughout the cytoplasm (Figure 2.10) (Brown and Benya 1988; Mallein-Gerin et al. 1991; Woods et al. 2005). Similarly, Li et al. (2006) have shown that spread chondrocytes exhibit long parallel actin fibres, in contrast to spherical-shaped chondrocytes which display disorganised short actin filaments. Numerous in vitro studies have also reported a similar difference in actin cytoskeleton organisation in round and spread morphologies for other cell phenotypes (Mooney et al. 1995; Doornaert et al. 2003; Tan et al. 2003; Engler et al. 2004; Yeung et al. 2005). Previous studies have claimed that the apparent material properties of the cell increased during cell spreading, resulting in the measurement of increased forces during mechanical loading (Caille et al. 2002; McGarry and McHugh 2008; McGarry 2009). However, the current chapter reveals that such experimentally observed “stiffness increases” in spread cells results in part from higher levels of formation of the actin cytoskeleton throughout the cell cytoplasm. The active modelling formulation implemented in the present chapter accurately captures the relationship between actin cytoskeleton distribution and cell spreading,
and subsequently demonstrates that the remodelling and contractility of the actin cytoskeleton leads to the increase in detachment forces during applied shear.

In the present chapter, the cell-substrate interface is simulated using a passive cohesive zone model and assumes a uniform adhesion strength at the cell base for both the 3 hr and 6 hr cell geometries. However, previous *in vitro* studies have shown cell-substrate adhesion is non-uniform, with focal adhesions clustering at the cell periphery (Chrzanowska-Wodnicka and Burridge 1996; Chen et al. 2003). Additionally, focal adhesions have been shown to be mechanosensitive, with active cell contractility and external loading being linked to focal adhesion assembly. For example, in the study of Parker et al. (2002) cells were seeded on micro-patterned islands and it was demonstrated that focal adhesions rearranged to regions where the highest tractions were measured. A study by Tan et al. (2003) has shown that regulating the number of focal adhesion complexes controls the traction exerted by cells on micro-posts. Additionally, the application of a mechanical force at the edge of cells using a micropipette resulted in focal adhesion formation (Riveline et al. 2001). In relation to the results of the current chapter, the higher level of formation of the actin cytoskeleton for the 6 hr cell will lead to higher levels of contractility, and hence focal adhesion formation. This interdependence of contractility and focal adhesion formation could result in further increases in the detachment resistance of spread cells. Computationally, Deshpande et al. (2008) have proposed a framework in which the active contractility of the actin cytoskeleton is coupled with traction induced focal adhesion assembly. This active biomechanical model has demonstrated the ability to accurately predict experimentally observed focal adhesion formation distribution for cells seeded on patterned substrates (Pathak et al. 2008). A three dimensional implementation of this framework has been developed by Ronan et al. (2012) to simulate cell spreading on elastic substrates. A framework incorporating active actin cytoskeleton remodelling and focal adhesion assembly has the potential to further elucidate the biomechanical phenomena governing cell adhesion. Furthermore, an extension of this framework to incorporate the kinetics of bond rupture should be developed in a follow-on study to further improve the prediction of detachment forces for round and spread cells.
The current chapter demonstrates that the pattern of bond rupture predicted during shear detachment is distinctly different for the 3 hr and 6 hr cells. Additionally, debonding for the 6 hr cell is dependent on the strength of the cell-substrate interface. Ofek et al. (2009) has shown that focal adhesion distribution is predominantly located at the back edge of chondrocytes during applied shear. Future experimental studies should consider the real-time visualisation of focal adhesion proteins such as vinculin and talin during applied shear in order to determine the exact pattern of bond rupture. Future studies could also examine the effect of probe height during shear detachment. In the experimental study of Huang et al. (2003), the probe was moved an arbitrary distance above the glass substrate prior to indentation. Simulations performed in the current chapter demonstrate that a probe height of 4 µm does not detach the 6 hr cell, which suggests that a low probe height was used in the study of Huang et al. (2003). Furthermore, the detachment of *in vitro* chondrocytes using micropipette aspiration would allow for a comparison of normal (mode I) cell detachment forces with the shear (mode II) detachment forces considered in the current chapter.

In conclusion, this chapter implements a 3D active modelling framework in order to investigate the increased resistance to detachment of spread chondrocytes as measured in the experimental study of Huang et al. (2003). Simulations illustrate that the larger adhesion area of the 6 hr cell results in a more highly developed actin cytoskeleton compared to the less spread 3 hr cell. Significant differences in cell detachment forces are computed between the 3 hr and 6 hr cell geometries, with predictions corresponding to experimental trends when the active modelling framework is used. It is also demonstrated that the detachment process for the 3 hr and 6 hr cells are considerably different, with initial bond rupture occurring at the front of the cell base for the 3 hr cell, compared to underneath the probe for the 6 hr cell. The current chapter reveals that the increased resistance to shear detachment of spread cells is due to the active contractile actin cytoskeleton, and provides new insight into the mechanisms governing cell adhesion.
References


### Tables

Table 6.1 Effect of seeding time on maximum adhesion force for single chondrocytes during shear indentation. All groups are statistically different from each other ($p < 0.05$). The values are reproduced from the study of Huang et al. (2003).

<table>
<thead>
<tr>
<th>Seeding Time (hrs)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Force (nN)</strong></td>
<td>34 ± 18</td>
<td>72 ± 14</td>
<td>183 ± 21</td>
<td>388 ± 78</td>
</tr>
</tbody>
</table>
Figures

Figure 6.1 (a) Plot of the shear traction – separation relationship for the cell-substrate interface. Finite element geometries for the: (b) 3 hr cell (c) 6 hr cell. The cytoplasm is shown in green and the nucleus is shown in blue. All dimensions are in μm. **Figure blurred due to copyright reasons.**
Figure 6.2 Contour plots of the average stress fibre activation levels ($\bar{\eta}$) in the cytoplasm after reaching a steady state equilibrium for the (a) 3 hr cell and (b) 6 hr cell. Contour plots of the variance ($\Pi$) in the cytoplasm after reaching a steady state equilibrium for the (c) 3 hr cell and (d) 6 hr cell. The nucleus and substrate are not shown in the images for clarity. A half-cell is shown due to symmetry. **Figure blurred due to copyright reasons.**
Figure 6.3 Contour plots of the average stress fibre activation levels ($\bar{\eta}$) at a probe indentation of 6 µm for the (a) 3 hr cell and (b) 6 hr cell. Contour plots of the variance ($\Pi$) at a probe indentation of 6 µm for the (c) 3 hr cell and (d) 6 hr cell. A probe height of 1 µm is simulated and probe movement is from right-to-left. The nucleus, probe and substrate are not shown in the images for clarity. A half-cell is shown due to symmetry. **Figure blurred due to copyright reasons.**
Figure 6.4 Contour plots of the average stress fibre activation levels ($\bar{\eta}$) for the 6 hr cell at a probe indentation of: (a) 3.71 µm; (b) 6.71 µm; (c) 13.24 µm. A probe height of 4 µm is simulated. A half-cell is shown due to symmetry. The simulation demonstrates that a probe height of 4 µm passes over the 6 hr cell without causing cell detachment. Figure blurred due to copyright reasons.
Figure 6.5 Probe force-indentation curves for the 3 hr (solid lines) and 6 hr (broken lines) cells with a passive hyperelastic cytoplasm and an active contractile cytoplasm. Predictions are shown for probe heights of 1 µm. Nodes on the base of the cell are fixed to the rigid substrate in order to prevent cell-substrate detachment for the above simulations. **Figure blurred due to copyright reasons.**
Figure 6.6 Probe force-indentation curves for the active model during the detachment of the: (a) 3 hr cell and (b) 6 hr cell. Peak detachment forces as a function of interface strength for both cell geometries are shown in (c). Interface strengths ($\tau_{max}$) simulated are: 0.05; 0.075; 0.1 and 0.2 kPa. Predictions are shown for a probe height of 1 $\mu$m. **Figure blurred due to copyright reasons.**
Figure 6.7 Contour plots of the traction ($\tau$) at the base of the 3 hr cell with an interface strength of 0.1 kPa at probe indentations of: (a) 6.89 $\mu$m; (b) 6.92 $\mu$m; (c) 6.96 $\mu$m; (d) 6.97 $\mu$m. The arrow indicates the direction of probe movement. Broken lines indicate the initial position of the base of the cell. **Figure blurred due to copyright reasons.**
Figure 6.8 Contour plots of the traction ($\tau$) at the base of the 6 hr cell with an interface strength of 0.05 kPa at probe indentations of: (a) 0.26 $\mu$m; (b) 2.10 $\mu$m; (c) 3.62 $\mu$m; (d) 5.17 $\mu$m. The arrow indicates the direction of probe movement. Broken lines indicate the initial position of the base of the cell. **Figure blurred due to copyright reasons.**
Figure 6.9 Contour plots of the traction ($\tau$) at the base of the 6 hr cell with an interface strength of 0.2 kPa at probe indentations of: (a) 11.15 μm; (b) 11.76 μm; (c) 12.86 μm; (d) 12.97 μm. The arrow indicates the direction of probe movement. Broken lines indicate the initial position of the base of the cell. Figure blurred due to copyright reasons.
7 In Situ Chondrocyte Deformation under Physiological Loading

7.1 Introduction

Numerous in vitro studies have demonstrated that the actin cytoskeleton plays a role in the biomechanical response of chondrocytes to mechanical stimuli (Trickey et al. 2004; Knight et al. 2006; Campbell et al. 2007; Ofek et al. 2009c). In addition, abnormal mechanical loads, which are believed to contribute to the development of osteoarthritis, can also affect the actin cytoskeleton (Blain 2009). Continuous high levels of hydrostatic pressure have been shown to alter the chondrocyte actin cytoskeleton such that it is similar to the actin cytoskeleton found in osteoarthritic chondrocytes (Fioravanti et al. 2005). However, the response of the actin cytoskeleton in chondrocytes in situ during physiological and abnormal mechanical loading is poorly understood.

Previous studies have experimentally determined local strains during cartilage-on-cartilage articulation (Wong et al. 2008b; Wong and Sah 2010b). In addition, Wong et al. (2008a) have shown that shear strain in degenerated cartilage is markedly increased near the surface due to both increased friction and a reduction in the mechanical properties of the tissue. A focal defect (FD) is characterised as a partial or full-thickness defect in a localised area of articular cartilage tissue, and is typically associated with acute injury or trauma (Hjelle et al. 2002; Widuchowski et al. 2007). Significantly, FDs have been shown to dramatically alter cartilage deformation during combined loading (Gratz et al. 2009; Wong and Sah 2010a). However, these experimentally determined strains, for both healthy and degenerated cartilage, have not previously been used to investigate the biomechanical response of chondrocytes. Understanding the biomechanical response of chondrocytes to abnormal strains is particularly important given the negative effect of high strain impact loads on chondrocyte activity (Quinn et al. 2001).

Finite element modelling has previously been used to investigate the stress state and deformation in chondrocytes during mechanical loading. Chondrocytes have been commonly modelled as biphasic and isotropic in multi-scale models when simulating their response to deformation (Guilak and Mow 2000; Han et al. 2007;
Han et al. 2011; Moo et al. 2012). As previously discussed in Chapters 2 and 5, viscoelastic models have been employed to simulate chondrocyte behaviour during single cell micropipette aspiration (Baaijens et al. 2005; Trickey et al. 2006) and shear experiments (McGarry and McHugh 2008). However, these models of chondrocytes have considered the cell as a passive, homogeneous material.

The objective of the current chapter is to investigate the role of the active remodelling of the actin cytoskeleton in the response of chondrocytes and the surrounding extracellular matrix (ECM) to physiological loading conditions. A 3D unit cell (UC) for cartilage tissue is created, comprising of a chondrocyte, surrounded by a pericellular matrix, and embedded in an extracellular matrix. An active bio-chemo-mechanical model based on the remodelling and contractility of the actin cytoskeleton (Deshpande et al. 2006) is implemented in order to simulate the biomechanical behaviour of chondrocytes. Physiological strain fields are chosen based on the experimental study of Wong and Sah (2010a) (Figure 7.1). Abnormal strains (Wong and Sah 2010a), due to the presence of a FD, are also simulated. Additionally, simulations are also performed which investigate the effect of the pericellular matrix (PCM) and cartilage anisotropy on chondrocyte biomechanics and actin cytoskeleton remodelling. It is demonstrated that the distribution and remodelling of the actin cytoskeleton plays an important role for in situ chondrocytes during abnormal loading. The work performed in this chapter has been published in Acta Biomaterialia (Dowling, E. P., Ronan, W., and McGarry, J. P. (2012). "Computational investigation of in situ chondrocyte deformation and actin cytoskeleton remodelling under physiological loading." Acta Biomaterialia, DOI:10.1016/j.actbio.2012.12.021).

7.2 Methods

7.2.1 Material Models and Properties

The active cell model parameters used in the current chapter are based on the previous calibration performed in Chapter 5, in which it was demonstrated that the active model can accurately predict response of single chondrocytes to in vitro mechanical shear deformation. The parameters are: $T_{max} = 0.85$ kPa, $\bar{k}_v = 6$, $\dot{e}_0 = 0.003$ s$^{-1}$, $\theta = 70$ s, $k_f = 1$; $k_b = 10$; $E_{cyto} = 1.5$ kPa; and $\nu_{cyto} = 0.4$. The nucleus is,
again, represented using a Neo-Hookean hyperelastic formulation. The Young’s modulus of the nucleus is assumed to be $E_{nuc} = 4$ kPa and the Poisson’s ratio is assumed to be $\nu_{nuc} = 0.4$ (Leipzig and Athanasiou 2008; McGarry and McHugh 2008; Ofek et al. 2009b).

For the majority of the simulations presented in the current study, the ECM is considered to be isotropic and homogeneous, and is modelled using a neo-Hookean hyperelastic formulation. A description of the neo-Hookean hyperelastic formulation is provided in the Chapter 4. Both the PCM and nucleus are considered to be isotropic and homogeneous, and are modelled using a neo-Hookean hyperelastic formulation. The stiffness of the PCM is assumed to be approximately one order of magnitude higher than the chondrocyte, and between 1-2 orders lower than the ECM (Guilak and Mow 2000; Alexopoulos et al. 2005b; Guilak et al. 2005). The Young’s modulus of the PCM is chosen to be $E_{PCM} = 15$ kPa (Guilak et al. 2005; Nguyen et al. 2010), unless specifically stated otherwise. Based on previous studies, the Young’s modulus of the ECM is chosen to be $E_{ECM} = 1.1$ MPa (Athanasiou et al. 1995; Pierce et al. 2009). The Poisson’s ratio is 0.4 for the ECM (Jin and Lewis 2004) and 0.125 for the PCM (Guilak and Mow 2000).

In addition to considering the ECM as an isotropic material, a number of simulations in the present study treat the ECM as an anisotropic material, based on the work of Pierce et al. (2009). The cartilage ECM is known to be anisotropic due to the arrangement of the Type II collagen fibres within the proteoglycan/water gel matrix. In the surface zone, fibres are orientated tangentially to the articular surface. The middle zone has fibres that are randomly orientated with the deep zone containing fibres that are orientated perpendicular to the surface. The anisotropic material behaviour of the ECM, incorporating collagen fibre orientation, is modelled using the Holzapfel-Gasser-Ogden strain energy potential in Abaqus (2012). A detailed description of the Holzapfel-Gasser-Ogden strain energy potential is provided in Chapter 4. The material constants for the anisotropic hyperelastic formulation are chosen based on previous studies (García and Cortés 2007; Pierce et al. 2009), with $C_{10} = 0.2085$ MPa, $C_{20} = 1.02 \times 10^{-3}$, $k_1 = 0.425$ MPa, $k_2 = 39.8$. Only one family of fibres ($N = 1$) is considered, and fibres are assumed to be perfectly aligned, thus $\kappa = 0$. Collagen fibre directions are defined in the global coordinate
system, and three mean preferred fibre directions ($\theta$) are considered: 0° (horizontally aligned fibres); 45°; and 90° (vertically aligned fibres), where $\theta$ is the angle between the fibre direction and x-axis in the global Cartesian coordinate system.

### 7.2.2 Unit Cell and Finite Element Implementation

A 3D unit cell (UC) of cartilage tissue is created, comprising of a chondrocyte cell, surrounded by a PCM, and embedded in a cuboidal ECM (Figure 7.2). The UC is generated in Abaqus 6.10. The chondrocyte is assumed to spherical, with a diameter of 16 µm, based on confocal images of in situ chondrocytes in previous studies (Idowu et al. 2000; Quinn et al. 2005; Sasazaki et al. 2008). Similarly, the nucleus is also chosen to be spherical, with a diameter of 7 µm (Guilak 1995; Knight et al. 2002; Ofek et al. 2009a), and the PCM is chosen to be 3 µm thick (Guilak and Mow 2000; Julkunen et al. 2009; Moo et al. 2012). A side length of 60 µm for the cuboidal ECM is chosen in order to give an approximate representation of chondrocyte volume per unit cartilage volume. This was determined by considering that the volume fraction of chondrocytes in cartilage tissue varies from 1-10% (Stockwell 1987; Quinn et al. 2005), and cellularity decreases with tissue depth from $150 \times 10^6$ cells/cm$^3$ at the surface to $50 \times 10^6$ cells/cm$^3$ at 500 µm below the surface (Jadin et al. 2005). Additionally, the ratio of cell volume to ECM volume in the current study is comparable to previous finite element models incorporating chondrocytes embedded in an ECM (Guilak and Mow 2000; Han et al. 2007; Korhonen et al. 2011; Moo et al. 2012).

Periodic boundary conditions, similar to those used by Mullins et al. (2007), are applied to the UC in order to ensure that opposing faces remain parallel during deformation. The periodic boundary conditions consist of a series of equation constraints, and can be expressed in terms of the nodal displacement vector, $\mathbf{u}$. For example, the nodes contained in the node set $\mathbf{u}_s$ remain parallel to the corresponding nodes in the node set $\mathbf{u}_p$, and $\mathbf{u}_b$ remains parallel to $\mathbf{u}_a$ during the simulation (Figure 7.3 (a) and (b)). Further details of the numerical implementation of the periodic boundary conditions are provided in Appendix A at the end of the thesis. Loads are imposed on the UC by applying a displacement boundary condition to the master nodes, $n_4$ and $n_5$, while $n_3$ is fully constrained. Boundary conditions are chosen to replicate the local cartilage strain fields observed experimentally by Sah.
and Wong (2010a) during physiological loading conditions, as described in section 7.2.3.

The cartilage tissue model is modelled using 8-noded linear brick elements (C3D8). The average element length for the nucleus, cytoplasm, PCM, and ECM is 0.27 µm, 0.48 µm, 0.88 µm and 1.62 µm respectively. A mesh sensitivity study was performed and no changes to the results were observed for smaller element sizes.

7.2.3 Strain-controlled Loading

All simulations are divided into two distinct phases. The first phase simulates the formation of an equilibrium actin cytoskeleton distribution in the cell in the absence of externally applied loading. As previously mentioned, an exponentially decaying signal in the cytoplasm (as observed for chondrocytes (Roberts et al. 2001)) drives the formation of the actin cytoskeleton, while tension reduction in the cytoplasm leads to dissociation. The second phase then involves the application of nominal strains to the UC. In the experimental study by Wong and Sah (2010a), local shear, axial and lateral strains were determined during articulation of intact rectangular blocks of bovine patellar articular cartilage as a function of normalised tissue depth. Additionally, a full-thickness FD was created in the centre of separate patellar cartilage samples. In the case of the tissue containing a FD, strain was also measured during articulation as a function of lateral distance from the edge of the defect. Nominal strains are applied in terms of the compressive, lateral and shear components measured by Wong and Sah (2010a) (Table 7.1) with respect to a reference global coordinate system. Plotting of the deformed shape in this global coordinate system facilitates the visualisation of the rotation and alignment of the cells with respect to the loading direction. Strain fields could also be applied in terms of the maximum and minimum principal strains, and the principal direction (Table 7.1). Nominal strain fields are applied as boundary conditions to the UC to simulate cartilage loading in the superficial, middle, and deep zones in fully intact healthy cartilage. In addition, simulations are performed for cartilage containing a FD, with the distance from the FD also being considered (Defect: Edge, Defect: Middle, Defect: Far). The same UC geometry is used to replicate intact cartilage and cartilage containing a FD, with only the strains applied (in the form of displacement boundary conditions) varying.
While the experiments of Wong and Sah (2010a) examined static loading conditions, both static and dynamic loading cases are considered in the present study (Figure 7.3 (c)). The effect of dynamic loading on the cellular level is investigated computationally in the current study as previous research has demonstrated the benefits of dynamic loading for cartilage homeostasis (Mauck et al. 2003). For static loading, nominal strains are applied for 0.5 seconds. In the case of dynamic loading, nominal strains are applied at frequency of 1 Hz, which is within the range of normal adult cadence (Waters et al. 1988). Again, strain values based on the study of Wong and Sah (2010a) are chosen as the dynamic loading cases, with the exception of simulations investigating the effect of the PCM and cartilage anisotropy, in which strain values are based on the study of Wong and Sah (2010b) (Table 7.1).

7.3 Results

7.3.1 Actin Cytoskeleton Distribution

An exponentially decaying signal leads to the formation of the actin cytoskeleton. Tension is actively generated by stress fibres, which leads to deformation of the cytoplasm. This cytoplasm deformation leads to a shortening of stress fibres, and an associated tension drop, as described in Eqn. 4.39, which in turn causes a partial dissociation of the actin cytoskeleton. The predicted distribution of the actin cytoskeleton (as described by the average actin intensity level $\bar{\eta}$) in response to the exponentially decaying signal is shown in Figure 7.4 (a). A steady state solution is predicted following 500 seconds, after which no further changes are computed. At this point, the cell is in equilibrium and all stress fibres in the actin cytoskeleton are at isometric tension with a strain rate of zero in every fibre direction. The actin cytoskeleton is predicted to be uniformly distributed throughout the cytoplasm due to the surrounding stiff PCM ($E_{ECM} = 15$ kPa) and ECM ($E_{PCM} = 1.1$ MPa), which prevents significant tension reduction in the cytoplasm. There is a slightly lower concentration in the actin cytoskeleton near the nucleus ($E_{nuc} = 4$ kPa) as the nucleus is less stiff than the PCM, and hence supports less stress fibre tension. The uniform distribution suggests that the actin cytoskeleton is smeared in appearance, without distinctive bundling of stress fibres in a dominant direction.
Chapter 7

7.3.2 Physiological and Abnormal Strain Loading

Following the simulation of the equilibrium distribution of the actin cytoskeleton under static conditions, the UC is subjected to physiological strain fields via the application of displacement boundary conditions. For example, Figure 7.4 (b) shows the maximum principal strain in the deformed UC during combined loading corresponding to the surface zone of intact cartilage (see Table 7.1). It can be noted that strains in the cell (~21-25%) are far higher than strains in the ECM (~1–5%). Figure 7.4 ((c) - (e)) shows the computed chondrocyte cell morphology at peak deformation following the application of experimentally determined strain fields at a range of tissue depths (the nucleus, PCM, and ECM are removed from the figures for clarity). The distribution of the actin cytoskeleton (\(\bar{\eta}\)) in the deformed cytoplasm is also shown for each case. The applied loading of the UC results in considerable cell deformation, which decreases with tissue depth. Additionally, applied loading results in the shortening of stress fibres and causes a tension reduction in the cell cytoplasm, which leads to a decrease in actin cytoskeleton concentration in all cases. Specifically, the following can be noted:

- At the surface zone (Figure 7.4 (c)), the cells in the FD case are significantly more flattened and elongated compared to the cell in the intact case. Additionally, cells in the FD case undergo less rotation compared to the cell in the intact case. The greatest level of actin cytoskeleton dissociation in the superficial zone is predicted for the cell in the intact case.

- In the middle zone (Figure 7.4 (d)), the cells in the FD case display a more elliptical shape compared to the cell in the intact case. All cells have a similar alignment. In addition, cells in the FD case have a greater level of actin cytoskeleton intensity. Stretching of the cell results in more fibres in the actin cytoskeleton remaining in a state of isometric tension, and this prevents dissociation. The highest values of \(\bar{\eta}\) are predicted in regions above and below the cell nucleus in the FD case.

- In the deep zone (Figure 7.4 (e)), the cell in the intact case is predicted to have a rounded morphology, whereas cells in the FD case display an elliptical shape. Additionally, the cell in the intact case experiences no rotation during loading. Cells in the FD case have a similar actin cytoskeleton distribution compared to
cells in the middle zone. In contrast to the superficial and middle zones, the cell in the intact case in the deep zone has a lower level of actin cytoskeleton dissociation compared to cells in the FD case.

### 7.3.3 Stresses in the Cell

Figure 7.5 shows the range of equivalent von Mises stress computed in the nucleus and cytoplasm following the application of experimentally determined strain fields for each case. As expected, the largest values of stress are predicted to occur in the surface zone, for both the nucleus and cytoplasm (Figure 7.5 (a)). Importantly, the maximum stress in both the nucleus and cytoplasm in the intact case is approximately 2-2.5 times lower than the maximum values computed for all the FD cases. A similar trend is predicted for stresses in the middle (Figure 7.5 (b)) and tidemark (Figure 7.5 (c)) zones. Typically, the largest values of nucleus and cytoplasm stress are computed for the regions closest to the FD. The aspect ratio is defined as the ratio of cell/nucleus length in the x-axis direction to cell/nucleus length in the y-axis direction. The change in aspect ratio of both the nucleus and cytoplasm at peak deformation, normalised with respect to the initial aspect ratio, is shown in Figure 7.6. In the surface zone (Figure 7.6 (a)), the nucleus and cytoplasm aspect ratio is approximately 3-4 times larger for the FD cases compared to the intact case. Similarly, the nucleus and cytoplasm aspect ratio in the middle (Figure 7.6 (b)) and deep (Figure 7.6 (c)) zones are larger when a FD is present. Simulations are also performed whereby cell shape and orientation was investigated. A horizontally-orientated ellipsoidal cell with an initial aspect ratio of 2, and a vertically-orientated ellipsoidal cell with an initial aspect ratio of 0.5 are considered. The aspect ratio of both the nucleus and cytoplasm for ellipsoidal cells fields at peak deformation is shown in Figure 7.7. For the horizontally-orientated ellipsoidal cell (‘flattened’ cell), the nucleus aspect ratio is approximately 1.5 times larger for the FD cases compared to the intact case in the surface zone (Figure 7.7 (i)). However, this difference in aspect ratio is reduced in the cytoplasm. In the middle zone (Figure 7.7 (ii)), this trend is reversed, with the difference in the aspect ratio for the intact and FD cases being larger in the cytoplasm compared to the nucleus. As shown in Figure 7.7 (iii), the nucleus and cytoplasm aspect ratios are slightly larger when a FD is present. For the vertically-orientated ellipsoid cell (‘columnar’ cell) in the surface zone, only the
region furthest away from the FD has a nucleus and cytoplasm aspect ratio greater than the intact case (Figure 7.7 (i)). The presence of a FD in the middle (Figure 7.7 (ii)) and deep (Fig. D-2B (iii)) zones results in a marginal change in the aspect ratio in the nucleus and cytoplasm compared to the intact case.

### 7.3.4 Dynamic Loading

Following the single loading results presented in Figures 7.4-7.7, the response of the UC to dynamic loading is considered (Figure 7.8). 30 loading cycles are implemented for a strain of $E_{xy} = 0.025, E_{yy} = -0.12, E_{xx} = 0.001$ at a frequency of 1 Hz. For the purposes of comparison, a static loading case is also considered in which the UC is loaded to the maximum experimentally observed strain, and held at this strain for a period of 29.5 seconds. Figure 7.8 (a) shows the change in the averaged actin cytoskeleton concentration in the cytoplasm as a function of time during dynamic and static loading. During dynamic loading, stress fibres undergo cyclic shortening and this leads to cyclic tension reduction in the cytoplasm. This cyclic tension drop causes gradual but continuous actin dissociation, in accordance with Eqn. 4.39. In contrast, there is only one applied load in the case of static loading; hence, the reduction in actin cytoskeleton concentration is much lower, reaching a plateau at approximately 20 seconds. Following the application of the single load, changes in stress and cytoskeletal remodelling occur over the subsequent 20 seconds before an equilibrium state is reached. After 20 seconds, all fibres in the actin cytoskeleton are at isometric tension, with no further dissociation of the actin cytoskeleton being computed, in accordance with Eqn. 4.39. As illustrated in Figure 7.8 (a) and (b), actin dissociation throughout the cell cytoplasm is far more pronounced in the case of dynamic loading than in the case of static loading after 30 seconds. The cyclic reduction in tension and subsequent low actin cytoskeleton concentration is predicted to affect the stress in the nucleus. As shown in Figure 7.8 (d) and (e), the equivalent von Mises stress computed in the nucleus in the dynamic loading case is approximately 1.5 times higher than in the static loading case at peak deformation. As shown in Figure 7.9, the equivalent von Mises stress computed in the nucleus in the static case reaches a peak value of 2.5 kPa when the UC is loaded to the maximum experimentally observed strain at 0.5 seconds. The stress then decreases, and reaches a plateau of approximately 0.9 kPa as the UC is held at the
maximum strain for 29.5 seconds. Similar to the static case, the equivalent von Mises stress predicted in the nucleus in the dynamic case reaches a peak value of 2.5 kPa at 0.5 seconds. During the subsequent loading cycles, the stress computed in the nucleus in the dynamic loading case is approximately 1.5 times higher than in the static case. During the unloading cycles, there is very little stress computed in the nucleus.

In Figure 7.8, signalling and formation of the actin cytoskeleton during cyclic loading was not considered. Interestingly, an experimental study by Roberts et al. (2001) has shown that an exponentially decaying signal is induced by the application of compressive loading to chondrocytes. Additionally, it has been demonstrated that cyclic compression of chondrocytes in agarose constructs induces disassembly of the actin cytoskeleton (Knight et al. 2006). Figure 7.10 shows the change in the normalised averaged actin cytoskeleton concentration in the cytoplasm as a function of time when an exponentially decaying signal is initiated at the start of each dynamic loading cycle, as motivated by the experimental observations of Roberts et al. (2001). As previously stated, the evolution of the actin cytoskeleton is dependent on the competition between signal-driven fibre formation and mechanically-driven fibre dissociation. Net disassembly of the actin cytoskeleton, as experimentally observed by Knight et al. (2006), is predicted if the model parameter \( \tilde{k}_b > \tilde{k}_f \). The earlier calibration of this modelling formulation (Chapter 5) for in vitro chondrocytes suggests that \( \tilde{k}_f = 1 \) and \( \tilde{k}_b = 10 \) provides an accurate prediction of the actin cytoskeleton distribution.

The effect of a FD on cell behaviour under dynamic loading is considered in Figure 7.11. A strain of \( E_{xy} = 0.025, E_{yy} = -0.12, E_{xx} = 0.001 \) is applied for the intact case, and \( E_{xy} = 0.06, E_{yy} = -0.2, E_{xx} = 0.08 \) for the FD case at a frequency of 1 Hz for 10 loading cycles. As shown in Figure 7.11 (a), in the intact case the cell exhibits a rounded morphology, with continuous actin dissociation predicted throughout the cytoplasm following five and ten loading cycles. The cell in the FD case displays a flattened, elongated shape with continuous actin dissociation predicted during cyclic loading (Figure 7.11 (b)). However, differences exist in the distribution of the actin cytoskeleton between the intact and FD cases. Dynamic loading reveals a greater decrease in \( \bar{\eta} \) above and below the nucleus for the cell in the FD case compared to
the intact case. Additionally, the sides of the cell in the FD case are considerably stretched, which results in less actin dissociation in these regions compared to the intact cell.

7.3.5 The Effect of the PCM

In all the simulations discussed above, the stiffness of the PCM is $E_{PCM} = 15$ kPa. In order to investigate the role of the PCM in chondrocyte biomechanics, simulations are performed whereby the stiffness of the PCM is varied, and dynamic compressive loading is performed ($E_{yy} = -0.225$ at 1 Hz for 10 loading cycles). Three values for the PCM stiffness ($E_{PCM}$) are considered: 5 kPa, 15 kPa and 750 kPa. When the cell is attached to a very stiff PCM ($E_{PCM} = 750$ kPa), prior to the application of loading, stress fibre tension is supported, thus leading to regions of high actin cytoskeleton concentration at the periphery of the cell. This is not the case when the cell is attached to a more compliant PCM ($E_{PCM} = 5$ kPa). Prior to the application of loading, the compliant PCM layer allows for high levels of cell deformation under the contractile action of the actin cytoskeleton. This in turn leads to a shortening of stress fibres, and an associated drop in stress fibre tension, in accordance with Eqn. 4.40. This tension drop causes stress fibres to dissociate, resulting in a lowered actin cytoskeleton concentration, in accordance with Eqn. 4.39. Hence, prior to the application of external loading, a significantly reduced actin cytoskeleton occurs when a compliant PCM surrounds the chondrocyte. Subsequent cyclic loading results in a reduction of the actin cytoskeleton intensity for all values of PCM stiffness. However, as shown in Figure 7.12 (a), the level of actin cytoskeleton intensity remains the highest in the case where the cell is surrounded by the PCM with the highest elastic modulus ($E_{PCM} = 750$ kPa).

The role of the PCM in regulation of cell deformation in response to external loading is considered in Figure 7.12 (b). If the PCM is significantly less stiff than the ECM, the outer deformation of the cell-PCM structure is determined by the deformation of the ECM. Additionally, within the cell-PCM structure, the cell deformation and hence the cell stress is governed by the cell-PCM stiffness ratio. This is evident from Figure 7.12 (b) (i) and (ii), where the outer deformation of a PCM of stiffness $E_{PCM} = 5$ kPa is very similar to that of a PCM of $E_{PCM} = 15$ kPa. However, in the latter case higher deformation of the cell is computed as the PCM is
significantly stiffer than the cell. In the former case the PCM deforms significantly more than for the case of Figure 7.12 (b) (i), hence less deformation of the cell is computed, with lower intracellular stresses being observed. If the PCM is of comparable stiffness to the ECM, then the overall deformation of the cell-PCM structure is influenced by the PCM-ECM stiffness ratio. As an example, Figure 7.12 (b) (iii) considers an ECM stiffness of $E_{\text{PCM}} = 750\text{kPa}$, which is comparable to the stiffness of the ECM chosen in the current study ($E_{\text{ECM}} = 1.1 \text{ MPa}$). In this case, the outer deformation of the cell-PCM structure is significantly lower than the predictions for the more compliant PCMs considered in Figure 7.12 (b) (i) and (ii). Hence, the overall cell stress is lower, despite the stiffness of the PCM being increased.

### 7.3.6 ECM Anisotropy

All the results presented so far have assumed that chondrocytes are embedded in an isotropic ECM. In Figure 7.13, the effect of cartilage anisotropy on chondrocyte behaviour and ECM stress is investigated following a single application of compressive ($E_{yy} = -0.225$) or shear ($E_{xy} = 0.25$) strain to an UC containing collagen fibres aligned at either $0^\circ$, $45^\circ$, or $90^\circ$ with respect to the x-axis in the global coordinate system. As shown in Figure 7.13 (a) (i), anisotropy has little effect on nucleus and cytoplasm stress during compression ($E_{yy}$). This is because the maximum principal strain is not sufficiently large for the fibres to make a significant contribution to the ECM mechanical response. However, a slight increase in maximum equivalent von Mises stress is computed in the nucleus if fibres are aligned in the maximum principal direction ($0^\circ$). For shear ($E_{xy}$), fibres must be orientated in the direction of max principal strain ($45^\circ$) in order to produce a noticeable change in nucleus stress (Figure 7.13 (a) (ii)). Anisotropy has a minor effect on the distribution of the actin cytoskeleton following dynamic pure compression or shear loading, as shown in Figure 7.13 (b) and (c) respectively. The stress state predicted in the ECM following applied compressive or shear strain is shown in Figure 7.13 (d) and (e) respectively. Under a compressive load, a higher stress is predicted in the ECM when fibres are aligned in the direction of maximum principal strain ($0^\circ$). If fibres are aligned in the direction of minimum principal strain ($90^\circ$), no fibre contribution is computed and ECM stress is similar to that predicted.
for an isotropic ECM. Similarly, during pure shear loading, the fibres must be orientated in the direction of maximum principal strain (45°) in order to generate a significant difference in ECM stress.

7.4 Discussion

The current chapter presents a computational investigation of the role of the remodelling and contractility of the actin cytoskeleton in the response of chondrocytes to physiological and abnormal (FD) strain loading. To the author’s knowledge, no previous computational model has considered the response of the actin cytoskeleton of chondrocytes during physiological loading, despite experimental studies showing that the actin cytoskeleton is linked to cartilage pathology (Capin-Gutierrez et al. 2004; Fioravanti et al. 2005; Lyman et al. 2012). In the current study, a chondrocyte cell embedded in an ECM was simulated using a 3D UC. An active modelling framework (Deshpande et al. 2006), incorporating signal dependent stress fibre formation and tension-dependent stress fibre dissociation, was used to predict the distribution and remodelling of the chondrocyte actin cytoskeleton during strain-controlled loading. In the current chapter, simulations elucidate the important role of the actin cytoskeleton during combined compressive, shear and lateral strain loading. In particular, the current chapter uncovers the following key findings: (i) the introduction of a FD significantly affects cellular deformation, increasing the stress experienced by the nucleus and cytoplasm, and altering the distribution of the actin cytoskeleton; (ii) dynamic loading results in a continuous decrease in actin cytoskeleton concentration but static loading does not; (iii) the stiffness of the PCM in the computational model significantly affects the concentration of the actin cytoskeleton in the cytoplasm; (iv) collagen fibre alignment affects cartilage behaviour in certain loading conditions. In the following sections, each of the above key findings (i-iv) will be discussed with reference to supporting experimental observations from the literature, highlighting the insights offered by the novel simulations presented in this chapter.

The current chapter demonstrates that chondrocyte biomechanics is considerably affected by altered strains due to the presence of a FD. Clinical studies have demonstrated that FDs can lead to cartilage degeneration, defect enlargement and tissue volume loss (Wang et al. 2006; Davies-Tuck et al. 2008; Wluka et al.
In addition, the abnormal strains that occur due to the presence of a FD are likely to make the tissue more susceptible to mechanical injury. Numerous experimental studies have examined the effect of abnormal mechanical loads, resulting in damaged cartilage, on chondrocyte viability and biosynthetic activity. It has been shown that mechanical injury to cartilage explants results in chondrocyte apoptosis with a loss of glycosaminoglycans (D’lima et al. 2001) and changes in gene expression (Lee et al. 2005). Similarly, Borelli Jr. et al. (2003) have shown that *in vivo* chondrocyte apoptosis can be caused by a single impact load. Furthermore *in vitro* chondrocyte necrosis can be induced by non-physiological stimuli (Kurz et al. 2005). Such alterations in cell behaviour may be related to the findings of the present study that abnormal strains result in a significant increase in cell deformation and stress during loading. It is important to note that abnormal loads and non-physiological stimuli may directly result in mechanical injury to cartilage tissue in the absence of a FD. However, the alteration in the mechanical environment of the tissue due to abnormal strains may initiate a similar biomechanical or biochemical cascade whether a FD is present or not, resulting in cell death and negatively impacting the biosynthetic activity of the tissue.

The present chapter demonstrates that actin cytoskeleton remodelling and contractility in chondrocytes is significantly altered in the presence of FDs. Changes in the distribution of the actin cytoskeleton in the chondrocyte cytoplasm are predicted for cells in the surface, middle and deep zones compared to the corresponding zones in the intact case. As previously stated, the actin cytoskeleton is very important not just for chondrocyte biomechanics, but also for cartilage homeostasis (Capin-Gutierrez et al. 2004; Fioravanti et al. 2005; Lyman et al. 2012). Hence, it is essential that chondrocyte models include a description of the mechano-biochemistry of the actin cytoskeleton, as implemented in the current study for the first time for chondrocytes during physiological loading.

The current chapter predicts that the stress in the nucleus is affected by the presence of a FD, and is highly dependent on the rate of actin cytoskeleton dissociation (dynamic loading and PCM stiffness are shown to alter nucleus stress). Chondrocyte nucleus deformation has been shown to affect gene expression (Buschmann et al. 1996). Also, impact-induced chondrocyte death has been linked
with the breakdown of DNA (Kühn et al. 2004). Nucleus deformation in chondrocytes has been investigated using passive material models (Guilak and Mow 2000; McGarry 2009). However, Chapter 5 demonstrates that an active contractility and remodelling framework for the actin cytoskeleton must be used to accurately predict the response of chondrocytes to in vitro shear loading, highlighting the deficiencies of passive modelling. Additionally, it is demonstrated in Chapter 4 that the nucleus stress is significantly affected by the contractile actin cytoskeleton.

The active modelling framework used in the present study predicts that dynamic loading will lead to more dissociation of the actin cytoskeleton compared to a single static load. Disruption of the actin cytoskeleton by the chemical cytochalasin-D has been shown to play an important role in maintaining the chondrocyte phenotype in mature chondrocytes (Newman and Watt 1988). The current study predicts that cyclic tension reduction, which occurs during dynamic loading, leads to the continuous dissociation of the actin cytoskeleton, suggesting that dynamic loading aids the preservation of chondrocyte phenotype. Dissociation of the actin cytoskeleton in chondrocytes has been shown to occur experimentally during cyclic compression (Knight et al. 2006; Campbell et al. 2007) and dynamic osmotic loading (Chao et al. 2006). The dynamic loading of single chondrocytes (Shieh and Athanasiou 2007) and chondrocytes embedded in agarose gel (Buschmann et al. 1995) has been shown to result in an upregulation of biosynthetic activity, while static loading leads to a reduction. Additionally, Haudenschild et al. (2008) have reported that both activation of the Rho pathway and remodelling of the actin cytoskeleton by dynamic compression, results in a change in gene expression in chondrocytes. Furthermore, a study by Sauter et al. (2012) has demonstrated that disruption of the actin cytoskeleton prevents necrotic chondrocyte death during impact loading. Understanding how dynamic loading conditions leads to the remodelling of the actin cytoskeleton provides vital insight into the biomechanisms involved in chondrocytes mechanotransduction.

The findings of present chapter provide an enhanced understanding of the link between applied loading, cytoplasm stress and actin cytoskeleton dissociation. Previous experimental observations suggest that this link is important in the maintenance of the chondrogenic phenotype and for cartilage tissue engineering.
strategies. Dissociation of the actin cytoskeleton has been shown to play a critical role in stimulating chondrogenic differentiation in mesenchymal stem cells (Zanetti and Solursh 1984; Lim et al. 2000). This would suggest that in order to induce the chondrocyte phenotype in mesenchymal stem cells, dynamic loading and hence continuous actin cytoskeleton dissociation is required. Additionally, dynamic loading has been demonstrated to be important for the generation of stem cell-based engineered cartilage constructs (Huang et al. 2010; Bian et al. 2012). The current approach for the design of tissue-engineered cartilage constructs is focused on parameters such as cell source, scaffolds and mechanical stimulation (Kock et al. 2011). However, several difficulties exist with these particular strategies. For example, chondrocyte expansion in monolayer or in 3D can cause de-differentiation, characterised by the formation of organised stress fibre bundles (Li et al. 2006), and a decrease in collagen type II expression (Darling and Athanasiou 2005). The 3D active modelling framework presented in the current chapter has the potential to benefit the tissue engineering of functional cartilage, using an approach such as identifying optimal mechanical loading conditions that prevents chondrocyte de-differentiation through prediction of actin cytoskeleton distribution.

The current chapter predicts that lowering the stiffness of the PCM allows for a reduction of tension at the edge of the cell and localised dissociation of the actin cytoskeleton in the cytoplasm. Experimental data suggests that the PCM stiffness is in the region of 15 – 100 kPa (Guilak et al. 2005; Darling et al. 2010; Nguyen et al. 2010; Wilusz et al. 2012). Hence, the stiffness of the PCM is considerably lower than the ECM, but is significantly higher than the effective stiffness of the active cell. This suggests that slight increases in PCM stiffness will increase the stress in the cell, up to the limit where the PCM stiffness becomes comparable to that of the ECM. The study of Choi et al. (2007) reports that the PCM can act as a protective layer for the chondrocyte in the superficial zone, and as a transducer that amplifies cellular strains in the middle and deep zones of cartilage tissue. These apparently differing effects of the PCM may be explained by the results of the present study, whereby the cell-PCM-ECM stiffness ratios determine if the PCM increases or decreases cell deformation and intracellular stress. Poole (1997) reported that the PCM reduces cell deformation during mechanical joint loading, which suggests that in these experiments the PCM is significantly less stiff than the ECM. A study by
Knight et al. (1998) has shown that isolated chondrocytes deform more than cells surrounded by a partially-formed PCM when embedded in 3% agarose gel with a stiffness of 25 kPa. Additionally, negligible deformation of the PCM shell was observed during loading, suggesting that the PCM is stiffer than the agarose matrix. This further highlights the importance of considering the stiffness ratio of the PCM to the ECM, as well the stiffness ratio between the cell and PCM, when investigating the biomechanical role of the PCM. Changes in the structure of the PCM have been shown to modify chondrocyte morphology in osteoarthritic cartilage (Korhonen et al. 2011). Furthermore, it has been demonstrated that collagen VI, the dominant collagen type in the PCM, functions in protecting the chondrocyte from apoptosis (Peters et al. 2011). The present chapter shows that the PCM plays a role in stress transfer to the cell, with significant implications for cytoskeletal remodelling.

The findings that the PCM plays an important role in controlling chondrocyte deformation are supported by finite element models of chondrocytes surrounded by a PCM (Alexopoulos et al. 2005a; Kim et al. 2010; Appelman et al. 2011; Han et al. 2011). However, these computational models have relied on passive material formulations of the chondrocyte, ignoring the key biomechanical features underlying the formation, remodelling, and contractility of the actin cytoskeleton. As discussed above, it is critically important to incorporate the active actin cytoskeleton in chondrocytes for the accurate prediction of stresses in the cytoplasm and nucleus. Such modelling provides an improved prediction of the role of the PCM in chondrocyte deformation.

The simulations in the current chapter suggest that collagen fibres must be orientated in the maximum principal direction in order to considerably affect the stress computed in the ECM. In addition, unless the applied maximum principal strain is sufficiently large, collagen fibres will not significantly contribute to the mechanical behaviour of the ECM. Finite element modelling of articular cartilage has previously been used to investigate chondrocyte biomechanics during loading. Moo et al. (2012) modelled the response of embedded chondrocytes in cartilage tissue to confined compression using a biphasic model to represent the cell, with the ECM assumed to be isotropic. A transversely isotropic model for cartilage, surrounding a passive non-contractile isotropic elastic chondrocyte, has been
employed to analyse cell deformation during compression (Han et al. 2007). A viscoelastic fibre-reinforced constitutive model based on a convex strain-energy function has been used to model collagen fibre and cartilage deformation during indentation, without explicitly modelling chondrocytes embedded in the tissue (Pierce et al. 2009; Pierce et al. 2010). The current chapter, for the first time, includes cells with a phenomenological collagen fibre model which is based on the Holzapfel-Gasser-Ogden strain energy potential to simulate both chondrocyte and cartilage tissue deformation. As previously stated above, chondrocytes actively respond to mechanical stimuli and the actin cytoskeleton plays an important role in governing this response. In order to offer insight or predictive capability into chondrocyte mechanotransduction, it is critical that finite element models of articular cartilage account for the active evolution of the actin cytoskeleton, rather than using passive material models.

A number of assumptions were made in the study and must be considered when interpreting the results. The viscoelastic behaviour of the PCM and ECM is not considered in the current model, and this may have implications for the actin cytoskeleton. During cyclic strain-controlled loading, stresses in a viscoelastic PCM-ECM would initially decrease, reaching a steady state based on the ratio of instantaneous to long-term moduli. The time to achieve steady state would be governed by the viscosity or relaxation time of the PCM-ECM material. Such “stress relaxation” of the PCM-ECM over time will result in lower steady state deformation of the cell, and this may slightly reduce the rate of dissociation of the actin cytoskeleton in response to cyclic loading. In the current study, the chondrocyte, PCM and ECM are assumed to perfectly bonded. Debonding of chondrocytes from the ECM/PCM during extreme loading could be investigated (McGarry et al. 2005). Furthermore, the current modelling framework could be used to investigate the response of chondrocytes to high strain rate shock loading (Jérusalem and Dao 2012).

For the further validation of predictions in the current chapter for chondrocytes embedded in an ECM experiencing physiological loading, visualisation of the actin cytoskeleton experimentally is required. In order to image the actin cytoskeleton in chondrocytes, current methods involve fixation,
permeablisation, and staining using antibodies. However, the actin cytoskeleton can rapidly remodel in response to mechanical stimuli, but the aforementioned techniques cannot provide real-time visualisation of chondrocyte actin dynamics. Future development must consider the real-time imaging of chondrocytes while undergoing deformation. A recent study by Haudenschild et al. (2009) demonstrates a technique for live-cell imaging of the chondrocyte actin cytoskeleton using lentiviral GFP-actin transduction for chondrocytes in agarose gel during compression.

In conclusion, a 3D active modelling framework incorporating actin cytoskeleton remodelling is implemented for the first time to simulate the response of embedded chondrocytes to physiological loading. The presence of a FD is shown to increase cellular deformation, altering the distribution of the actin cytoskeleton and increasing nucleus stresses. The current chapter predicts that dynamic loading leads to continuous dissociation of the actin cytoskeleton due to the cyclic tension reduction within the chondrocyte. Furthermore, it is demonstrated that the active evolution of the chondrocyte actin cytoskeleton is influenced by the stiffness of the PCM. The accurate prediction of cytoskeletal remodelling and nucleus stress in chondrocytes under physiological loading conditions offers the potential to guide strategies for cartilage tissue engineering.


Tables

Table 7.1 Nominal strain components applied to the UC based on the experimentally measured strains fields from the study of Wong & Sah (2010a). Femoral condyle and tibial plateau strain components are based on strains from Wong & Sah (2010b). Strains are also expressed in terms of the maximum ($E_{\text{max}}$) and minimum ($E_{\text{min}}$) principal strains, and the principal direction ($\phi$). **Table reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.**

<table>
<thead>
<tr>
<th>Surface</th>
<th>$E_{xy}$ (shear)</th>
<th>$E_{yy}$ (compression)</th>
<th>$E_{xx}$ (lateral)</th>
<th>$E_{\text{max}}$</th>
<th>$E_{\text{min}}$</th>
<th>$\phi$ (°)</th>
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<td>0.0262</td>
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<td>-0.1250</td>
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Figure 7.1 Experimentally measured shear (A, B), axial (D, E), and lateral (G, H) strain maps of patellar cartilage, either intact (A, D, G) or containing a focal defect (B, E, H), after articulation against trochlear cartilage. Local shear (C), axial (F), and lateral (I) strain versus normalised tissue depth for patella cartilage as intact, or with a focal defect (as a function of lateral distance from the defect edge (EDGE, MID, FAR)). Figure reproduced from Wong and Sah (2010a). **Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.**
Figure 7.2 Schematic of the 3D unit cell (UC) cartilage model consisting of a single chondrocyte cell surrounded by the PCM, and embedded in a cuboidal ECM (note: image is not to scale). The chondrocyte cytoplasm is modelled using an active actin cytoskeleton formulation in parallel with a hyperelastic material. The nucleus and PCM are modelled as a hyperelastic isotropic material. The ECM is modelled either as a hyperelastic isotropic or anisotropic material. Collagen fibres are included in the anisotropic ECM, with mean fibre directions ($\theta$) of 0°, 45° and 90° with respect to the x-axis in the global Cartesian coordinate system used in simulations. The schematic illustrates collagen fibres orientated at 0°. Half of the model is shown due to symmetry. All dimensions are in µm. **Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.**
Figure 7.3 Schematic of the periodic boundary conditions applied to the: (a) undeformed UC ($n_1, n_4, n_5,$ and $n_8$ refer to the corner nodes of the UC, $a, d, p,$ and $s$ correspond to node sets situated on a vertex of the UC); (b) deformed UC. (c) Illustration of the strain profile for static loading (left) and dynamic loading (right) at a frequency of 1 Hz. Strain profile is shown for a duration of three seconds. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.4 (a) Contour plot of the distribution of the actin cytoskeleton ($\bar{\eta}$) in the chondrocyte cytoplasm at 500 seconds after signal initiation. The PCM and ECM are not shown in (A) for clarity. (b) Deformed UC during loading. Contours illustrate the maximum principal strain in the cartilage tissue. Deformed cell geometry for the intact and FD cases following application of experimentally determined strain fields at the: (c) surface zone; (d) middle zone; (e) deep zone. The nucleus, PCM and ECM are not shown in (c), (d) and (e) for clarity. Contours illustrate the distribution of the actin cytoskeleton ($\bar{\eta}$) in the chondrocyte cytoplasm. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.5 Range of equivalent tensile stress (von Mises) computed in the nucleus (left) and cytoplasm (right) following application of experimentally determined strain fields for the intact and FD cases: (a) surface zone; (b) middle zone; (c) deep zone. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.6 Aspect ratio computed in the nucleus (left) and cytoplasm (right) following application of experimentally determined strain fields for the intact and FD cases: (a) surface zone; (b) middle zone; (c) deep zone. The initial aspect ratio of the spherical cell geometry before loading is one. Aspect ratios normalised with respect to the initial aspect ratio of the cell geometry are shown. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.7 Nucleus and cytoplasm aspect ratios computed in the ‘flattened’ cell (a) and ‘columnar’ cell (b) geometries following application of experimentally determined strain fields for the intact and FD cases. The initial aspect ratio of the ‘flattened’ cell and ‘columnar’ cell geometries before loading are 2 and 0.5 respectively. Aspect ratios normalised with respect to the initial aspect ratio of the cell geometry are shown. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.8 (a) The effect of static and dynamic loading on the averaged actin cytoskeleton intensity ($\bar{\eta}$) in the cytoplasm. Computed changes over 30 seconds are considered. An experimentally determined strain of $E_{xy} = 0.025$, $E_{yy} = -0.12$, $E_{xx} = 0.001$ at 1Hz was applied. Distribution of the actin cytoskeleton ($\bar{\eta}$) in the chondrocyte cytoplasm at peak deformation following 30 seconds of: (b) static and (c) dynamic loading. Note: contour limits in (b) are different to (c). The nucleus, PCM and ECM are not shown in (b) and (c) for clarity. Contour plots of the equivalent von Mises stress in the nucleus at peak deformation following 30 seconds of: (d) static and (e) dynamic loading. The PCM and ECM are not shown in (d) and (e) for clarity. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.9 Equivalent von Mises stress computed in the nucleus during static and dynamic loading. 30 loading cycles are implemented for a strain of $E_{xy} = 0.025$, $E_{yy} = -0.12$, $E_{xx} = 0.001$ at a frequency of 1 Hz. The static load reaches maximum strain at 0.5 seconds, and is held at this strain for a period of 29.5 seconds. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.10 The effect of cyclic-initiated signalling on the averaged actin cytoskeleton ($\bar{\eta}$) as a function of time in the cytoplasm, normalised with respect to the steady state averaged actin cytoskeleton ($\bar{\eta}_0$). Computed changes over 30 seconds are considered. Predictions are shown for four values of the non-dimensional dissociation rate constant ($\bar{k}_b$): 1; 10; 50; and 100. **Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.**
Figure 7.11 Deformed cell geometries during dynamic loading for the intact (left) and FD case (right). Contours illustrate the distribution of the actin cytoskeleton ($\bar{\eta}$) in the chondrocyte cytoplasm at: (a) peak deformation during the 5th cycle; (b) peak deformation during the 10th cycle. Note: contour limits change from the 5th to the 10th cycle. A strain of $E_{xy} = 0.025$, $E_{yy} = -0.12$, $E_{xx} = 0.001$ was applied for the intact case, and $E_{xy} = 0.06$, $E_{yy} = -0.2$, $E_{xx} = 0.08$ for the FD case. The nucleus, PCM and ECM are not shown for clarity. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Figure 7.12 The effect of PCM stiffness on: (a) actin cytoskeleton distribution in the cytoplasm; and (b) PCM, cell, and nucleus stress during cyclic compression loading (\(E_{yy} = -0.225\) at 1 Hz for 10 cycles). Deformed cell geometry at peak deformation during the 10th loading cycle is shown. Contours illustrate the actin cytoskeleton intensity (\(\bar{\eta}\)) and the equivalent von Mises stress in (a) and (b) respectively. The nucleus, PCM and ECM are not shown in (a) for clarity. The ECM is not shown in (b) for clarity. *Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.*
Figure 7.13 The effect of ECM anisotropy on cell and ECM stress, and actin cytoskeleton organisation during compressive ($E_{yy} = -0.225$) and shear ($E_{xy} = 0.25$) loading. (a) Range of equivalent von Mises stress computed in the nucleus and
cytoplasm following application of strain fields for the isotropic and anisotropic models. Fibres orientated at 0°, 45°, and 90° with respect to the x-axis in the global coordinate system are considered. Deformed cell geometry and actin cytoskeleton distribution ($\bar{n}$) in the cytoplasm is shown following ten cycles (1 Hz) of (b) compressive and (c) shear strain. Fibres aligned in the direction of maximum principal strain during compression (0°) and shear (45°) are illustrated for the anisotropic model. The nucleus, PCM and ECM are not shown in (b) and (c) for clarity. Range of equivalent von Mises computed in the ECM following applied (d) compressive and (e) shear strain. Fibres aligned in the direction of maximum principal strain during compression (0°) and shear (45°) are illustrated for the anisotropic model. The nucleus, cytoplasm and PCM are not shown in (d) and (e) for clarity. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
8 Discussion and Concluding Remarks

The aim of this final chapter is to present an overall discussion of the main findings of this thesis. Additionally, future perspectives for investigating chondrocyte biomechanics involving the approach used in this thesis are presented. The overall goal of this thesis is to gain a more thorough understanding of the role of the actin cytoskeleton in chondrocyte biomechanics by performing finite element simulations and \textit{in vitro} experiments. This combined modelling-experimental approach offers a novel insight into the effect of active contractility and remodelling of the actin cytoskeleton in the response of chondrocytes to mechanical loading.

Experimentally, chondrocyte biomechanics research has focused on investigating the mechanical properties of chondrocytes, in addition to examining the changes in the intracellular structure of the cell in response to mechanical stimuli. Investigators have carried out this research through the development of high-precision single cell testing systems. The experimental work performed in this thesis uses such a high-precision test system to apply shear deformation to individual chondrocytes, in conjunction with a video-capture method that monitors the deformed cell geometry and probe deflection. This allows for accurate measurements of the force required to deform the cell throughout the shear experiment to be determined. Additionally, alterations in the actin cytoskeleton during shear deformation were observed. Furthermore, the contributions of the three cytoskeleton components to the shear resistance of chondrocytes were established. This experimental work presents, for the first time, an investigation of the role of the cytoskeleton in the response of chondrocytes to shear. This work is particularly relevant to the field of chondrocyte biomechanics research, given the links between shear loading and cartilage pathology (Lee et al. 2002; Smith et al. 2004; Wong et al. 2008; Wong and Sah 2010). However, experimentation alone cannot fully reveal the underlying mechanisms governing the response of chondrocytes to mechanical loading. \textit{In vitro} experiments in tandem with computational modelling have the potential to elucidate these mechanisms. Finite element modelling has previously been used to simulate chondrocyte behaviour in response to applied loading and to characterise the mechanical properties of the cell. Previous models have considered the chondrocyte...
as a passive material, using either elastic, viscoelastic or biphasic material formulations to represent the cell (Baaijens et al. 2005; Trickey et al. 2006; McGarry and McHugh 2008; Ofek et al. 2009a; Nguyen et al. 2010). However, passive material models provide limited insight into cellular behaviour since the key mechanisms by which cells actively respond to mechanical stimuli are ignored. In this thesis, a 3D active modelling framework which incorporates the biomechanisms underlying the formation, dissociation and contractility of the actin cytoskeleton is implemented. Simulations using this active model reveal for the first time that the active contractile actin cytoskeleton plays a key role in the response of chondrocytes to shear deformation. Additionally, simulations uncover that the increased resistance to detachment of spread chondrocytes as experimentally measured by Huang et al. (2003) is due to increased formation of the actin cytoskeleton. Furthermore, the response of the actin cytoskeleton during physiological loading is simulated for the first time in this thesis. The combined experimental-computational approach presented in this thesis has yielded important observations regarding the response of chondrocytes to mechanical loading, which could not be demonstrated through experimentation alone, or through the use of passive material models.

In Chapter 5, in vitro shear experiments were performed on untreated chondrocytes, and on chondrocytes in which the cytoskeleton components were disrupted. A distinctive yield shape for the force probe-indentation curve was observed for untreated cells, while a linear curve was observed for cells in which the active contractile actin cytoskeleton was removed. Similarly, disruption of the actin cytoskeleton has been shown to alter the biomechanical response of chondrocytes to compression (Ofek et al. 2009b) and micropipette aspiration (Trickey et al. 2004). In contrast, disruption of the microtubules and intermediate filaments did not considerably affect the probe force-indentation curves. It was shown that the active modelling formulation accurately predicts the response of untreated chondrocytes to applied shear, demonstrating the ability of the active model to capture the distinctive yield point of the probe force-indentation curve. Furthermore, a reduction in the passive stiffness of the cytoplasm corresponds to chondrocyte behaviour when the intermediate filaments and microtubules are disrupted. Moreover, a passive hyperelastic model is shown to provide an accurate prediction only for cells in which
the actin cytoskeleton has been chemically disrupted. The work carried out in
Chapter 5 provides, for the first time, a quantification and interpretation of the
significance of the remodelling and contractility of the actin cytoskeleton in
chondrocytes during shear. The active model reveals that the tensile and compressive
strain imposed on different regions within the cell during shear loading results in
very different regimes of actin cytoskeleton behaviour. Prior to shear deformation,
the actin cytoskeleton is predominantly located at the base of the cell and
surrounding the nucleus. During applied shear, the cytoplasm at the front of the cell
is compressed into the substrate, leading to a shortening of the fibres in the actin
cytoskeleton in this region. As a result, there is a reduction in tension and localised
dissociation of the actin cytoskeleton is predicted. In contrast, at the back of the cell,
there is no reduction in tension and the actin cytoskeleton stretches at isometric
tension and remains intact. The yield point in the probe force–indentation curve, that
is demonstrated both experimentally and computationally, is a direct result of the
distinctive behaviour of the actin cytoskeleton in compressive and tensile regions of
the cell. It is established that this behaviour can only be predicted by an active
modelling framework that incorporates the key features of formation, dissociation
and contractility of the actin cytoskeleton.

The fluorescent images presented in Chapter 5 show that the chondrocyte
actin cytoskeleton contains no distinctive bundling of stress fibres. This corresponds
well with fluorescent images of primary chondrocytes reported in previous studies
(Durrant et al. 1999; Idowu et al. 2000; Langelier et al. 2000; Trickey et al. 2004;
Leipzig et al. 2006). In contrast, highly aligned bands of stress fibres have been
reported for other cell phenotypes (Wang et al. 2001; Tan et al. 2003; Kaunas et al.
2005; Thery et al. 2006; Pellegrin and Mellor 2007; Yang et al. 2007; Buxboim et al.
2010). The active modelling formulation used in this thesis has previously been used
to simulate the stress fibre distribution for smooth muscle cells, mesenchymal stem
cells and fibroblasts seeded on microposts (McGarry et al. 2009), and on continuous
flat rigid substrates (Ronan et al. 2012). It was shown that the stress fibre variance in
smooth muscles cells ($\Pi_{\text{max}} \approx 0.8$), mesenchymal stem cells ($\Pi_{\text{max}} \approx 0.8$), and
fibroblasts ($\Pi_{\text{max}} \approx 0.8$) is very high, predicting stress fibres become highly aligned
in dominant directions for these cells (Ronan et al. 2012). In contrast, the simulations
in Chapter 5 predict a low value for the stress fibre variance in the chondrocyte cytoplasm ($\Pi_{\text{max}} \approx 0.4$), indicating that highly aligned stress fibres bundles are not formed for chondrocytes, as observed experimentally. However, despite the absence of highly aligned distinctive stress fibres in chondrocytes, it is clearly demonstrated in the experiments and simulations of Chapter 5 that the smeared actin cytoskeleton provides a highly significant contribution to the biomechanical behaviour of chondrocytes. The active modelling framework predicts that the remodelling and contractility of the actin cytoskeleton causes stress in the cell nucleus, even in the absence of externally applied loading. In contrast, passive material models can only compute stresses in the nucleus due to externally applied loading, such as probe indentation. Subsequently, the predicted cytoplasm and nucleus stress state following probe indentation is significantly different between the active cell model and the passive cell model. The accurate prediction of nucleus stresses is particularly important in light of experimental studies demonstrating that nucleus deformation is closely related to gene expression (Maniotis et al. 1997; Roca-Cusachs et al. 2008; Thomas et al. 2009). The active biomechanical model used in this thesis offers significant potential towards uncovering the link between active contractility, nucleus deformation and gene expression.

In Chapter 6, the active modelling framework is implemented to determine whether the active contractility and remodelling of the actin cytoskeleton can account for the increased force required to detach highly spread chondrocytes from a substrate, as observed in the \textit{in vitro} study of Huang et al. (2003). It is demonstrated that the active formulation, combined with a cohesive zone model, provides a reasonable agreement with the measurements for a round cell seeded for three hours (3 hr cell) and a highly spread cell seeded for six hours (6 hr cell). Numerous finite element studies have used passive material models in order to capture the behaviour of spread cells in response to mechanical stimuli (Thoumine et al. 1999; Caille et al. 2002; McGarry and McHugh 2008; McGarry 2009). However, an artificial increase in the material properties of the cell is required in order to capture the experimentally measured increases in resistance to deformation. In particular, McGarry and McHugh (2008) used a viscoelastic material model for the chondrocyte cytoplasm to simulate the experimental findings of Huang et al. It was shown that an 18-fold
increase in cell stiffness in tandem with a 4-fold increase in the cell-substrate interface strength must be artificially implemented for spread chondrocytes in order to compute detachment forces similar to experimental values. In contrast, the simulations performed in Chapter 6 demonstrate that an interface strength of 0.2 kPa provides a reasonable prediction of the detachment forces for both the 3 hr and 6 hr cells. Importantly, the active modelling formulation uses an unchanged set of material parameters for both cell geometries (see next paragraph for further discussion on this important point). Chapter 6 provides a significant advancement on previous finite element models simulating chondrocyte detachment by demonstrating that there is no requirement to artificially adjust the material properties of the cell or the interface strength in order to capture the experimental measurements of Huang et al. (2003) for round and spread cells.

In Chapter 6, a more highly developed actin cytoskeleton is predicted for the more spread 6 hr cell geometry in comparison to the less spread 3 hr cell geometry. This is in agreement with experimental images, which show that round chondrocytes display a punctuate smeared actin cytoskeleton, without highly aligned stress fibres (Durrant et al. 1999; Idowu et al. 2000; Langelier et al. 2000; Trickey et al. 2004; Leipzig et al. 2006). In contrast, chondrocytes that have spread over several hours develop a flattened highly spread morphology, and exhibit distinctive bundling of stress fibres (Brown and Benya 1988; Mallein-Gerin et al. 1991; Leipzig et al. 2006; Li et al. 2006). Additionally, in the study of Huang et al. (2003), fluorescence microscopy showed that the actin cytoskeleton rearranged from the centre of the cell to the periphery following spreading. The 6 hr cell has a larger adhesion area and is less deformable geometry than the 3 hr cell, hence the 6 hr cell provides more support for cytoskeletal tension. As a result, higher level of actin cytoskeleton formation is predicted in the cell cytoskeleton using the active modelling framework. This computational prediction is closely aligned with the aforementioned experimental observations, highlighting the important role of cell morphology in actin cytoskeletal distribution. It is shown in Chapter 6 that the more highly formed actin cytoskeleton in the spread cell increases the force required to indent and detach the cell. Overall, the simulations performed in this chapter accurately capture the connection between cell spreading and actin cytoskeleton organisation, and
attachment force. These findings provide novel insight into the spreading and adhesion of cells, which is important for understanding fundamental cellular processes such as proliferation, differentiation and cell-signalling (Frenkel et al. 1996; Chen et al. 1997).

In Chapter 7, the response of the actin cytoskeleton of chondrocytes to experimentally determined physiological and abnormal strain fields (Wong and Sah 2010) is investigated. This is of particular relevance in light of experimental studies that demonstrate a link between the actin cytoskeleton and cartilage pathology (Capin-Gutierrez et al. 2004; Fioravanti et al. 2005; Lyman et al. 2012). Simulations in this chapter predict that the presence of a focal defect in cartilage considerably affects cellular deformation, causing the stress in the cytoplasm and nucleus to significantly increase, and altering the actin cytoskeleton distribution compared to that predicted for chondrocytes in healthy normal cartilage. Experimentally, it has been demonstrated that mechanical injury to chondrocytes and cartilage can lead to changes in gene expression (Buschmann et al. 1996; Kühn et al. 2004; Lee et al. 2005) and cell death (D'lima et al. 2001; Quinn et al. 2001; Kurz et al. 2005). Such experimentally observed changes in the behaviour of chondrocyte cells may be related to the finding of Chapter 7 that cell and nucleus deformation is significantly increased in the presence of a focal defect. The active modelling framework used in this thesis predicts that cyclic tension reduction, which occurs during dynamic loading, results in increased dissociation of the actin cytoskeleton compared to static loading. This finding can provide an interpretation of previously reported experimental observations for chondrocytes. For example, disruption of the actin cytoskeleton in chondrocytes during cyclic compression (Knight et al. 2006; Campbell et al. 2007) and dynamic osmotic loading (Chao et al. 2006) can be explained by the finding that cyclic shortening of stress fibres will lead to dissociation of the actin cytoskeleton. Additionally, disruption of the actin cytoskeleton has been demonstrated to be important for the regulation of the chondrocyte phenotype in mature chondrocytes (Newman and Watt 1988). The finding that cyclic loading leads to the continuous dissociation of the actin cytoskeleton suggests that dynamic loading may aid the preservation of the chondrocyte phenotype. This has important implications for understanding how
dynamic loading conditions lead to the remodelling of the actin cytoskeleton, and subsequent changes in gene expression. Shieh and Athanasiou (2007) have shown that the dynamic loading of single chondrocytes results in an up-regulation of biosynthetic activity, while static loading leads to a reduction. A similar observation has been reported for chondrocytes embedded in agarose gel (Buschmann et al. 1995). Additionally, it has been demonstrated that both activation of the Rho pathway and remodelling of the chondrocyte actin cytoskeleton by dynamic compression, results in a change in gene expression (Haudenschild et al. 2008a; Haudenschild et al. 2008b).

In Chapter 7 simulations were also performed to investigate the effect of the pericellular matrix (PCM) and cartilage tissue anisotropy on actin cytoskeleton remodelling and chondrocyte biomechanics. It is predicted that the PCM, which is significantly more compliant than the extracellular matrix (ECM), allows for a reduction in tension at the cell periphery and hence a localised dissociation of the actin cytoskeleton. The absence of the PCM leads to higher localised strains being imposed on to the cell via the stiff ECM. Numerous experimental studies have shown that the PCM plays an important biomechanical role by acting as a buffer of stresses applied on to the cell (Poole 1997; Knight et al. 1998; Peters et al. 2011). The finding that the PCM is important for controlling chondrocyte deformation is supported by finite element models including both the chondrocyte and the PCM (Alexopoulos et al. 2005; Kim et al. 2010; Appelman et al. 2011; Han et al. 2011). However, these models have relied on passive material formulations of the chondrocyte. As previously discussed, it is critically important to incorporate the actin cytoskeleton in chondrocytes for the accurate prediction of stresses in the cytoplasm and nucleus. Such active modelling offers an enhanced prediction of the role of the PCM in chondrocyte biomechanics. Additionally, it is demonstrated in Chapter 7 that modelling the anisotropy of the ECM is important for certain loading regimes. It is assumed that collagen fibres buckle under a compressive load, and only provide a structural contribution in tension. It is computed that cellular behaviour is significantly affected when fibres are orientated orthogonal to the loading direction during compression or in the direction of maximum shear. Overall, the findings of Chapter 7 are of particular importance for understanding the biomechanisms
underlying experimental observations such as actin cytoskeleton dissociation during the dynamic loading of chondrocytes, and abnormal strain loads causing cell necrosis and apoptosis.

Numerous experimental studies have demonstrated that the chondrocyte actin cytoskeleton appears at the periphery (cortex) of the cell, just beneath the plasma membrane, and is composed of punctate F-actin. In particular, a distinct ring of cortical actin, approximately 1-2 μm in thickness, forms in chondrocytes that are embedded in 3D constructs such as agarose or alginate gel (refer to Figures 2.7 and 2.8 (c) for fluorescent images) (Idowu et al. 2000; Knight et al. 2001; Knight et al. 2006; Campbell et al. 2007; Haudenschild et al. 2009; Haudenschild et al. 2011; Chen et al. 2012). Although the active cell contractility model not explicitly include a term to increase F-actin polymerisation around the membrane, the active model does predict, for a cell adhered to a rigid substrate, a higher intensity of the actin cytoskeleton at the periphery, near the cell base, compared to a lower intensity in the middle of the cell (Figure 5.7 (b)). Additionally, a quite thick cortical shell (approximately 3.5 μm) with a high actin cytoskeleton intensity is computed for a chondrocyte surrounded by a PCM and embedded in an ECM, which mimics the in situ environment (Figure 7.4 (a)). This is due to the PCM supporting stress fibre tension, thus leading to regions of high actin cytoskeleton concentration at the periphery of the cell. There is a lower concentration in the actin cytoskeleton in the regions of the cytoplasm surrounding the nucleus, as the nucleus is less stiff than the PCM, and hence supports less stress fibre tension.

The formation of cortical actin in chondrocytes may be influenced by how the cell senses and responds to the surrounding environment. It is known that actin organization in cells in a 2D environment is regulated by the mechanical properties of the substrate (Discher et al. 2005). A distinct cortical shell of F-actin may not form in chondrocytes with a round morphology that are adhered to stiff substrates. This may be due to the small adhesion area, which results in a considerable reduction in tension in the regions of the cytoplasm that are not close to the cell base, leading to the dissociation of the actin cytoskeleton. In contrast, for chondrocytes embedded in an ECM, either in vivo or in hydrogel constructs, the surrounding structure is
considerably stiffer than the effective stiffness of the cytoplasm and would prevent a drop in tension at the periphery of the cell, which may lead to the appearance of a cortical ring of actin. This implies that the stiffness of the surrounding ECM, and additionally the PCM stiffness, may play an important role in regulating the formation of cortical actin. In the study of Chen et al. (2012), cortical actin was demonstrated to be significantly greater for chondrocytes embedded in a stiffer material (3% agarose concentration compared to 1% agarose). Additionally, it has been shown that actin morphology in endothelial cells is significantly affected by the stiffness of collagen gels (Byfield et al. 2009). The compliancy of the nucleus may be also important in the formation of cortical actin. It is widely believed that the nucleus is considerably stiffer than the cytoplasm; however large nucleus deformations have been observed in experimental studies (Dahl et al. 2005; Pajerowski et al. 2007; Leipzig and Athanasiou 2008; Roca-Cusachs et al. 2008; Buxboim et al. 2010). This suggests that the shear and bulk modulus of the nucleus may be lower than the values reported in the literature, and nucleus stiffness may be comparable with that of the cytoplasm. This would allow for more nucleus deformation in the cell, leading to a reduction in tension in regions of the cytoplasm surrounding the nucleus, and causing dissociation of the actin cytoskeleton in those specific regions. Osmotic stress in cartilage and cell compressibility may also play a role in regulating the cortical actin cytoskeleton in chondrocytes. Mechanical loading of cartilage causes the exudation of interstitial water, which results in an increase in the concentration of negatively charged proteoglycans. This causes an increase in the swelling pressure in the tissue, which is then resisted by the collagen fibre network. The changes in proteoglycan concentration and swelling pressure are believed to contribute to the changes in cell shape and volume that are observed during mechanical loading (Guilak 1995; Guilak et al. 1995). Importantly, experimental studies have showed that osmotic loading induces a remodelling of the actin cytoskeleton in chondrocytes (Guilak et al. 2002; Erickson et al. 2003; Chao et al. 2006). Growth factors may also affect the cortical actin cytoskeleton. A study by Leipzig et al. (2006) demonstrated that the addition of TGF-β1 and IGF-I leads to a more distinct ring of cortical actin forming in chondrocytes compared to untreated cells. Similarly, the addition of TGF-β1 appears to promote the formation of cortical
actin for chondrocytes seeded on glass substrates for three hours, as shown in Appendix B, Figure B-1 (iii).

As previously discussed, the active modelling formulation implemented in this thesis has been thoroughly validated for *in vitro* single chondrocytes during applied shear. In order to further elucidate the biomechanical phenomena governing chondrocyte mechanotransduction, several experimental techniques should be considered, in parallel with active computational modelling. The approach undertaken in this thesis to image the actin cytoskeleton involved the fixation and permeabilisation of a single chondrocyte during mechanical loading, and subsequent staining using fluorescent antibodies. However, the actin cytoskeleton can rapidly remodel in response to mechanical stimuli, but the method used in this thesis does not provide real-time visualisation of chondrocyte actin dynamics. Additionally, a method for visualising the actin cytoskeleton within living cells using suitable cell-permanent fluorescent labels is currently unavailable. Future development must consider the live imaging of the actin cytoskeleton during cell deformation. Campbell and associates (Campbell et al. 2007; Campbell and Knight 2007) have developed a technique to investigate real-time actin dynamics both qualitatively and quantitatively by using an immortalised mature chondrocyte cell line transfected with an eGFP-actin plasmid in conjunction with a fluorescent recovery after photobleaching (FRAP) technique. Additionally, a study by Haudenschild et al. (2009) has demonstrated live-cell imaging of the chondrocyte actin cytoskeleton using lentiviral GFP-actin transduction for chondrocytes in agarose gel during compression. In addition to imaging of the actin cytoskeleton, enhanced imaging of focal adhesions should also be considered in future studies. Focusing on the interface between the cell and the surrounding environment, numerous *in vitro* studies have demonstrated a link between focal adhesions and active cell contractility (Riveline et al. 2001; Parker et al. 2002; Tan et al. 2003; Sniadecki et al. 2007). Fluorescent imaging of the focal adhesion protein vinculin was performed in this thesis for chondrocytes during applied shear, but only for probe indentations that did not lead to bond rupture between the cell and substrate. Future experimental studies should consider examining focal adhesion evolution during cell detachment. In terms of the computational modelling of the cell-substrate interface, simulations in this thesis use
a passive cohesive zone formulation. A biomechanical model in which the active contractility of the actin cytoskeleton is coupled with traction-induced focal adhesion assembly has been developed by Deshpande et al. (2008) and implemented in a 2D scenario by Pathak et al. (2008). A 3D framework incorporating active remodelling of the cytoskeleton and focal adhesion assembly would provide further insight into the biomechanical phenomena governing chondrocyte adhesion and detachment.

The 3D active biomechanical model presented in this thesis could be used to complement recently published studies that investigate chondrocyte biomechanics. The experimental results reported in the study of Chen et al. (2012) demonstrate that the level of cortical actin cytoskeleton organisation increases more rapidly for cells cultured in 3% agarose compared to cells in 1% agarose. The active modelling framework could be used to provide an interpretation of this experimental observation. A decrease in the stiffness of the agarose gel will result in reduced support for cytoskeletal tension in the embedded chondrocytes, hence the actin cytoskeleton will dissociate. This is analogous to the finding of Chapter 6 that the compliant PCM results in a lower level of cytoskeletal formation. Similarly, predictions of the remodelling of the chondrocyte actin cytoskeleton using the active model could provide insight into the mechanisms involved in the dissociation of the actin cytoskeleton during micropipette aspiration as reported by Pravincumar et al. (2012). Finally, a recently reported experimental technique developed to investigate in situ chondrocyte behaviour in response to static and dynamic loading (Herzog et al., 2012) reports changes in cell morphology. The implementation of in situ loading following actin filament disruption using a chemical agent such as cytochalasin-D could provide further insights and validation of the findings presented in this thesis.

The findings of this thesis provide an enhanced understanding of the link between applied loading, cytoplasm stress and actin cytoskeleton dissociation. Previous experimental observations demonstrate that dissociation of the actin cytoskeleton plays a role in stimulating chondrogenic differentiation in mesenchymal stem cells (Zanetti and Solursh 1984; Lim et al. 2000), and that dynamic loading is important for stem cell-based tissue engineering strategies (Huang et al. 2010; Bian et al. 2012). This would suggest that in order to induce the chondrocyte phenotype in
stem cells, dynamic loading and hence continuous actin cytoskeleton dissociation is required. Several difficulties exist with current strategies for designing tissue-engineered cartilage constructs, such as the failure of cells to maintain the chondrocyte phenotype (Darling and Athanasiou 2005; Li et al. 2006; Chung and Burdick 2008; Kock et al. 2011; Lee et al. 2011). The 3D active modelling framework presented in this thesis may benefit the tissue engineering of functional cartilage, offering the potential to identify optimal mechanical loading conditions to stimulate chondrogenic stem cell differentiation through prediction of actin cytoskeleton distribution and nucleus deformation.

In conclusion, this thesis entails the merging of a 3D active modelling framework, which incorporates actin cytoskeleton remodelling and contractility, with novel single cell experiments in order to gain a more in depth understanding of the role of the actin cytoskeleton in chondrocyte biomechanics. In vitro experiments reveal that untreated chondrocytes containing an actin cytoskeleton respond to an applied shear deformation in a fundamentally different manner to cells in which the actin cytoskeleton has been disrupted. Simulations using the active 3D framework uncover that this response is entirely due to the contractility and remodelling of the actin cytoskeleton. It is also demonstrated that passive hyperelastic models can only be used to predict the response of non-contractile chondrocytes. Additionally, it is revealed that the increased resistance to shear detachment of spread chondrocytes is due to a high level of actin cytoskeleton formation throughout the cytoplasm. Furthermore, it is demonstrated that the active evolution of the chondrocyte cytoskeleton in situ is affected by the presence of a focal defect in cartilage tissue. It is predicted that dynamic loading leads to continuous dissociation of the actin cytoskeleton due to cyclic reduction in tension within the cell cytoplasm. The combined modelling–experimental framework used in this thesis has provided novel insight into the role of the active contractility and remodelling of the chondrocyte actin cytoskeleton in response to mechanical loading. The findings of this thesis may have important implications for the understanding of the pathogenesis of cartilage tissue and the tissue engineering of cartilage constructs.
References


Appendix A

Periodic boundary conditions were applied to the cartilage tissue model to ensure that corresponding nodes on opposing faces of the unit cell (UC) undergo identical displacements. An equal number of nodes are required along the edges of opposite faces of the geometry in order to apply the periodic boundary conditions. The periodic boundary conditions consist of a series of equation constraints, and can be expressed in terms of the nodal displacement vector, \( \mathbf{u} \), such that:

\[
\begin{align*}
\mathbf{u}_F - \mathbf{u}_{n_4} &= \mathbf{u}_E \\
\mathbf{u}_B - \mathbf{u}_{n_5} &= \mathbf{u}_A \\
\mathbf{u}_D - \mathbf{u}_{n_2} &= \mathbf{u}_C
\end{align*}
\]  

(A1) 

(A2) 

(A3)

\[
\begin{align*}
\mathbf{u}_q - \mathbf{u}_{n_2} &= \mathbf{u}_p \\
\mathbf{u}_s - \mathbf{u}_{n_5} &= \mathbf{u}_p \\
\mathbf{u}_r - \mathbf{u}_6 &= \mathbf{u}_p \\
\mathbf{u}_x - \mathbf{u}_{n_5} &= \mathbf{u}_w \\
\mathbf{u}_y - \mathbf{u}_{n_8} &= \mathbf{u}_w \\
\mathbf{u}_z - \mathbf{u}_{n_4} &= \mathbf{u}_w \\
\mathbf{u}_b - \mathbf{u}_{n_4} &= \mathbf{u}_a \\
\mathbf{u}_d - \mathbf{u}_{n_2} &= \mathbf{u}_a \\
\mathbf{u}_c - \mathbf{u}_{n_3} &= \mathbf{u}_a
\end{align*}
\]  

(A4) 

(A5) 

(A6) 

(A7) 

(A8) 

(A9) 

(A10) 

(A11) 

(A12)

\[
\begin{align*}
\mathbf{u}_{n_7} - \mathbf{u}_{n_3} &= \mathbf{u}_{n_5} \\
\mathbf{u}_{n_6} - \mathbf{u}_{n_2} &= \mathbf{u}_{n_5}
\end{align*}
\]  

(A13) 

(A15)

\[
\begin{align*}
\mathbf{u}_{n_8} - \mathbf{u}_{n_4} &= \mathbf{u}_{n_5} \\
\mathbf{u}_{n_3} - \mathbf{u}_{n_4} &= \mathbf{u}_{n_2}
\end{align*}
\]  

(A14) 

(A16)

where the subscripts \( n_1 - n_8 \) refer to the corner nodes of the UC (Fig. A-1 (a)). The subscripts \( A, B, C, D, E, \) and \( F \) correspond to nodes sets located on the faces of the UC (as labelled in Fig. A-1 (b)), excluding any nodes situated on the vertices or corners of the UC. The subscripts \( a, b, c, d, p, q, r, s, w, x, y, \) and \( z \) correspond to node sets situated on each vertex of the UC, excluding nodes contained in the corner node sets \( n_1 - n_8 \) (Fig. A-1 (c)). For all simulations, the corner node \( n_1 \) is fully constrained such that \( \mathbf{u}_{n_1} = 0 \).
Appendix A

Figures

Figure A-1 Schematic for implementation of periodic boundary conditions on the UC. Figure reproduced with permission from Dowling et al. 2012, Acta Biomater, DOI:10.1016/j.actbio.2012.12.021.
Appendix B

To further investigate the role of the actin cytoskeleton in chondrocyte biomechanics, experiments were performed where in vitro single chondrocytes were mechanically compressed by using a flat-ended probe. Additionally, chondrocytes were treated with cyto-D or a growth factor (TGF-β1), and then statically compressed using a probe. The effect of TGF-β1 was studied as it has been reported (Leipzig et al. 2006; Koay et al. 2008) that TGF-β1 increases the levels of F-actin in the cytoplasm, and increases the resistance of cell to compressive deformation.

The creep cytoindentation apparatus (CCA) was used to apply a constant compressive stress to individual adherent cells, while measuring the resultant cell deformation (Koay et al. 2008). The apparatus consists of the following components: a force transducer (cantilever), a sensor (laser micrometer), a motor (piezoelectric translator), and a controller (PC running LabVIEW 6i). Chondrocytes were seeded using a similar protocol to that described in Chapter 3. Media contained either cytochalasin-D (Cyto-D, 2 μM) or TGF-β1 (10 ng/ml). After 3 hours of culture, slides were placed into the CCA for single-cell compression. A single chondrocyte was selected for compression and fixation. Before compression, the cell diameter and position of the cell were recorded. A tungsten probe of diameter 50 μm was positioned above the cell. The probe was lowered until a load of 75 nN was applied to the cell. 3.7% paraformaldehyde in PBS (warmed to 37 °C) was placed in the dish, and compression was begun immediately. Cells were fixed for 10 minutes and held under compression for the entire duration. Then the probe was raised off the cell and the microscope slide was removed for staining as described in Chapter 3.

Differences in morphology and intracellular structure were observed between cells treated with cyto-D and TGF-β1. Cyto-D broke down actin microfilaments into spotty aggregates. However a distinct ring of actin was observed at the periphery of cells containing TGF-β1. Following compression, cytochalasin cells were highly deformed and flattened. Deformation also caused the nucleus to change morphology.
and very few focal adhesions were observed. TGF-β1 treated cells were also deformed, but only to a slight extent compared to Cyto-D treated cells. Additionally, a distinct actin cytoskeleton and focal adhesion assembly was still apparent. Although the experimental focus of this thesis is the shear deformation of chondrocytes, the representative images of chondrocytes under compression illustrated below support the finding that actin cytoskeleton remodelling and nucleus deformation is important during the response of chondrocytes to mechanical loading.
References


Figure B-1 Immunocytochemistry images showing alterations in cell structure in response to a 75 nN compressive load. The bright field image, nuclei (displayed in blue), actin network (displayed in red), and focal adhesions (displayed in green) are shown for a representative: (i) cyto-D control and (ii) cyto-D compressed cell; (iii) TGF-β1 control and (iv) TGF-β1 compressed cell at focal planes close to the base of the cell. **Figure blurred due to copyright reasons.**